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The Role Of Protein Tyrosine Phosphatase 1b In The Central Regulation Of Energy Homeostasis

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Abstract
Protein tyrosine phosphatase 1B (PTP1B) is a ubiquitously expressed tyrosine phosphatase implicated in the central control of energy homeostasis via negative regulation of leptin signaling. Mice with central nervous system (CNS)-specific PTP1B deficiency demonstrate clear metabolic improvements, including decreased body weight and adiposity presumably due to enhanced leptin sensitivity. Interestingly, compound PTP1B:leptin double mutants show attenuated weight gain compared to leptin single mutants, suggesting that the metabolic effects of PTP1B deficiency may also involve non-leptin signaling pathways. Indeed, insulin and other non-leptin cytokines, such as interleukin-6, have been implicated in the CNS control of energy balance, and these pathways are also candidates of PTP1B regulation. Thus, whether or not the metabolic effects of PTP1B deficiency are due exclusively to enhanced leptin signaling remains unclear. This dissertation examines the role of central PTP1B in the control of leptin receptor-dependent energy homeostasis. A leptin receptor-expressing cell specific PTP1B-deficient (LepRb PTP1B /) mouse model was generated and its metabolic phenotype was analyzed in comparison to wildtype controls and to whole body PTP1B knockouts. Though subtle phenotypic differences between LepRb-PTP1B-/- and PTP1B-/- mice exist, LepRb-PTP1B-/- mice demonstrate a majority of the phenotype observed in whole body PTP1B knockouts, including decreased body weight and adiposity and improved leptin sensitivity compared to wildtype controls. To further elucidate whether or not central PTP1B regulates non-leptin signaling pathways, a compound hypothalamic PTP1B:LepRb double mutant (Nkx2.1-PTP1B-/-:LepRb-/-) was generated and metabolically phenotyped in comparison to hypothalamic leptin receptor (Nkx2.1-LepRb-/-) and PTP1B (Nkx2.1-PTP1B-/-) mutant mice. While Nkx2.1-PTP1B-/- mice show decreased body weight and adiposity compared to wildtypes, both Nkx2.1-PTP1B-/-:LepRb /- and Nkx2.1-LepRb-/- show a severe obese phenotype marked by similarly increased weight gain, total fat mass, food intake, and glucose intolerance, indicating that the metabolic benefits of hypothalamic PTP1B deficiency are dependent upon functional leptin receptor signaling. Finally, whether PTP1B can regulate interleukin-6 signaling was explored using an immortalized mouse hypothalamic cell line. In summary, these data show that PTP1B is a critical regulator of energy balance within leptin receptor-expressing cells and within the hypothalamus specifically, and further begin to unravel the signaling pathways mediating the beneficial metabolic effects of central PTP1B deficiency.

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ABSTRACT

THE ROLE OF PROTEIN TYROSINE PHOSPHATASE 1B IN THE CENTRAL REGULATION OF ENERGY HOMEOSTASIS

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Kendra K. Bence

Protein tyrosine phosphatase 1B (PTP1B) is a ubiquitously expressed tyrosine phosphatase implicated in the central control of energy homeostasis via negative regulation of leptin signaling. Mice with central nervous system (CNS)-specific PTP1B deficiency demonstrate clear metabolic improvements, including decreased body weight and adiposity presumably due to enhanced leptin sensitivity. Interestingly, compound PTP1B:leptin double mutants show attenuated weight gain compared to leptin single mutants, suggesting that the metabolic effects of PTP1B deficiency may also involve non-leptin signaling pathways. Indeed, insulin and other non-leptin cytokines, such as interleukin-6, have been implicated in the CNS control of energy balance, and these pathways are also candidates of PTP1B regulation. Thus, whether or not the metabolic effects of PTP1B deficiency are due exclusively to enhanced leptin signaling remains unclear. This dissertation examines the role of central PTP1B in the control of leptin receptor-dependent energy homeostasis. A leptin receptor-expressing cell specific PTP1B-deficient (LepRb-PTP1B-/-) mouse model was generated and its metabolic phenotype was analyzed in comparison to wildtype controls and to whole body PTP1B knockouts. Though subtle phenotypic differences between LepRb-PTP1B-/- and PTP1B-/- mice exist, LepRb-PTP1B-/- mice demonstrate a majority of the phenotype observed in whole body PTP1B knockouts, including decreased body weight and adiposity and improved leptin sensitivity compared to wildtype controls. To further elucidate whether or not central PTP1B regulates non-leptin signaling pathways, a compound hypothalamic PTP1B:LepRb double mutant (Nkx2.1-PTP1B-/-:LepRb-/-)

was generated and metabolically phenotyped in comparison to hypothalamic leptin receptor (Nkx2.1-LepRb-/-) and PTP1B (Nkx2.1-PTP1B-/-) mutant mice. While Nkx2.1-PTP1B-/- mice show decreased body weight and adiposity compared to wildtypes, both Nkx2.1-PTP1B-/-:LepRb-/- and Nkx2.1-LepRb-/- show a severe obese phenotype marked by similarly increased weight gain, total fat mass, food intake, and glucose intolerance, indicating that the metabolic benefits of hypothalamic PTP1B deficiency are dependent upon functional leptin receptor signaling. Finally, whether PTP1B can regulate interleukin-6 signaling was explored using an immortalized mouse hypothalamic cell line. In summary, these data show that PTP1B is a critical regulator of energy balance within leptin receptor-expressing cells and within the hypothalamus specifically, and further begin to unravel the signaling pathways mediating the beneficial metabolic effects of central PTP1B deficiency.

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CHAPTER 1: Introduction

Obesity is a widespread and increasing health problem within the United States and worldwide (Flegal et al., 2012; Popkin et al., 2012; Popkin and Slining, 2013). Based upon recent estimates from the 2009-2010 National Health and Nutrition Examination Survey within the United States, more than one third of the U.S. population is classified as obese (Body Mass Index, BMI≥30), and approximately 70% of the total population is defined as overweight or obese (BMI≥25) (Flegal et al., 2012). With its persistent rise in both youth and adult populations and its associated comorbidities with type 2 diabetes, hypertension, and certain cancers (esophageal, pancreatic, breast), obesity has enormous public health consequences (Ahima, 2006). Furthermore, no effective, long-term treatment for obesity exists. Dieting and exercise are often insufficient in maintaining sustained weight loss, whereas more invasive options such as bariatric surgery are not cost-effective for treating the greater population (Stefater et al., 2012b). Thus, understanding the underlying biological mechanisms which regulate body weight and adiposity is of great importance and may lead to new insights for more effective treatment options.

Although obesity is predominantly thought to be a disease affecting the periphery (i.e. increased fat mass deposition), the central nervous system (CNS) plays a key role in the regulation of appetite, body weight, and metabolism. Neurons within the hypothalamus integrate neuroendocrine signals from the periphery, detecting both short and long term energy status. These neurons project to downstream hypothalamic and extrahypothalamic nuclei, and the activity of these homeostatic circuits can elicit changes in feeding or energy expenditure, ultimately leading to changes in body weight. Numerous cell signals play a role in the central control of body weight, and disruption of cellular signaling pathways within the neurons underlying energy balance can promote obesity.

Phosphorylation is an important regulatory mechanism of these intracellular signaling pathways; the opposing actions of kinase and phosphatase activity determine the phosphorylation status of signaling components. Indeed, metabolically relevant signaling pathways such as leptin and insulin require several tyrosine phosphorylation events for downstream signal transduction. While the action of protein tyrosine kinases (PTKs) initiating signaling cascades is fairly well understood, relatively less is known regarding the role of protein tyrosine phosphatases (PTPs) in these pathways. PTPs can negatively regulate signaling pathways by dephosphorylating phosphotyrosyl residues, but in some instances may actually act as positive regulators of signaling. Gaining a better understanding of the role of CNS PTPs in the control of energy balance is thus essential and may highlight PTPs as ideal therapeutic targets for sensitizing signaling pathways disrupted in the obese state. One such pathway is the neuroendocrine leptin signaling pathway.

Leptin and the neuroendocrine control of body weight

Leptin is a 16 kDa protein secreted by adipose tissue and is found at circulating levels in proportion to total body fat (Frederich et al., 1995). It was first discovered in 1994 through cloning of the mouse *ob* gene (Zhang et al., 1994). Mice with mutations in leptin (*ob/ob*) are obese and exhibit diabetic symptoms (Mayer et al., 1953; Garthwaite et al., 1980; Lindström, 2007). Additional studies found that mice lacking leptin receptors (*db/db* or *Lepr*-/-) were similarly obese, resulting from increased food intake and decreased energy expenditure (Friedman and Halaas, 1998). Like *ob/ob* mice, human leptin deficiency results in obesity (Montague et al., 1997), and leptin replacement therapy has been shown to rescue the obese phenotype of genetic leptin deficiency in both mice and humans (Farooqi et al., 1999). Exogenous intraperitoneal leptin administration decreased food intake and body weight of both *ob/ob* and wild type mice, and central intracerebroventricular administration also reproduced the food intake and body weight reduction, suggesting leptin had direct effects within the brain (Campfield et al., 1995; Halaas et al., 1995).

Leptin acts through its receptor (LepR), encoded by the *Lepr* gene in the mouse. Identified in 1995, the LepR belongs to the class I cytokine superfamily. At the time, only LepR with a short intracellular domain was identified and cloned (LepRa). However LepRa was shown to be closely related to the gp130 signaling-transducing component of the IL-6 receptor, the leukemia inhibitory factor (LIF) receptor, and the granulocyte colony-stimulating factor (G-CSF) receptor – all class I cytokine receptors containing long intracellular domains, suggesting the possibility of alternatively spliced forms of the leptin receptor (Tartaglia et al., 1995). In fact, at least six alternatively spliced isoforms of the LepR have since been identified in mice (Lee et al., 1996; Margetic et al., 2002). The short form receptor, LepRa, is thought to play a role in transport or uptake of circulating leptin. In contrast, the long form of the receptor, LepRb, contains a 320 amino acid cytoplasmic domain, capable of activating downstream intracellular signaling cascades (Bjørbaek and Kahn, 2004; Leshan et al., 2006). Within the CNS, LepRb is expressed in various regions of the brain. LepRb is expressed in a number of specific hypothalamic nuclei including the arcuate nucleus, ventromedial hypothalamus, dorsomedial hypothalamus, lateral hypothalamic area, and ventral premammillary nucleus (Mercer et al., 1996; Elmquist et al., 1998; Scott et al., 2009; Patterson et al., 2011). Additionally, LepRb is expressed in many extrahypothalamic areas including the cortex, cerebellum, hippocampus, thalamus, nucleus accumbens, ventral tegmental area, parabrachial nucleus, and hindbrain (Elmquist et al., 1998; Scott et al., 2009; Patterson et al., 2011).

Despite broad central expression of the leptin receptor, the main effects of leptin on energy homeostasis have focused on the hypothalamus. Indeed, the hypothalamus has the highest number and concentration of leptin receptors (Patterson et al., 2011), and early rat lesion studies implicated this brain region in the central control of energy balance (Brobeck et al., 1943; Hetherington, 1944; Leibowitz et al., 1981). Within the arcuate nucleus, leptin acts upon two subpopulations of neurons defined by which neuropeptides they express (Elias et al., 1998;

Korner et al., 1999). The two groups of neurons express either the anorectic peptide precursor pro-opiomelanocortin (POMC) or the orexigenic peptides neuropeptide Y (NPY) and Agouti-related protein (AgRP). POMC is cleaved into alpha melanocyte-stimulating hormone (α-MSH), and stimulation of POMC neurons by leptin increases expression of *Pomc* transcript, POMC neuron excitability, and release of neurotransmitters, including α-MSH (Cowley et al., 2001). α-MSH can then act on downstream melanocortin receptors inhibiting energy intake and increasing energy expenditure (De Jonghe et al., 2011b). Conversely, leptin stimulation inhibits both AgRP and NPY expression and hyperpolarizes AgRP/NPY neurons (Stephens et al., 1995; Glaum et al., 1996; Schwartz et al., 1996; Elias et al., 1999). It is this dual action on two opposing neuron populations that allows leptin to elicit its physiological effects of reducing food intake and body weight. Leptin resistance is thought to underlie the obese disease state as obese individuals have increased circulating leptin levels without the corresponding reductions in food intake or body weight (Myers et al., 2008, 2010).

Leptin signaling is tyrosine phosphorylation dependent

At the cellular level, leptin binding to LepRb results in the activation of the associated tyrosine kinase Janus kinase 2 (JAK2). JAK2 autophosphorylates and phosphorylates several tyrosine residues along the intracellular tail of the LepRb including Y985, Y1077, and Y1138 (Bjørbaek et al., 1997; Li et al., 1999). Phosphorylation of Y985 allows for recruitment of tyrosine phosphatase SHP2 and activation of extracellular signal-regulated kinase (ERK) 1/2 pathway, while phosphorylation of Y1077 and Y1138 recruits the transcription factors signal transducer and activator of transcription 5 and 3 (STAT5 and STAT3), respectively. Activated STAT5 or STAT3 can then translocate to the nucleus and regulate gene expression (White et al., 1997; Banks et al., 2000) (Fig. 1.1). Thus, phosphorylation of Y985, Y1077, and Y1138 activates separate arms of the leptin signaling pathway, each contributing to leptin's metabolic effects in differing ways.

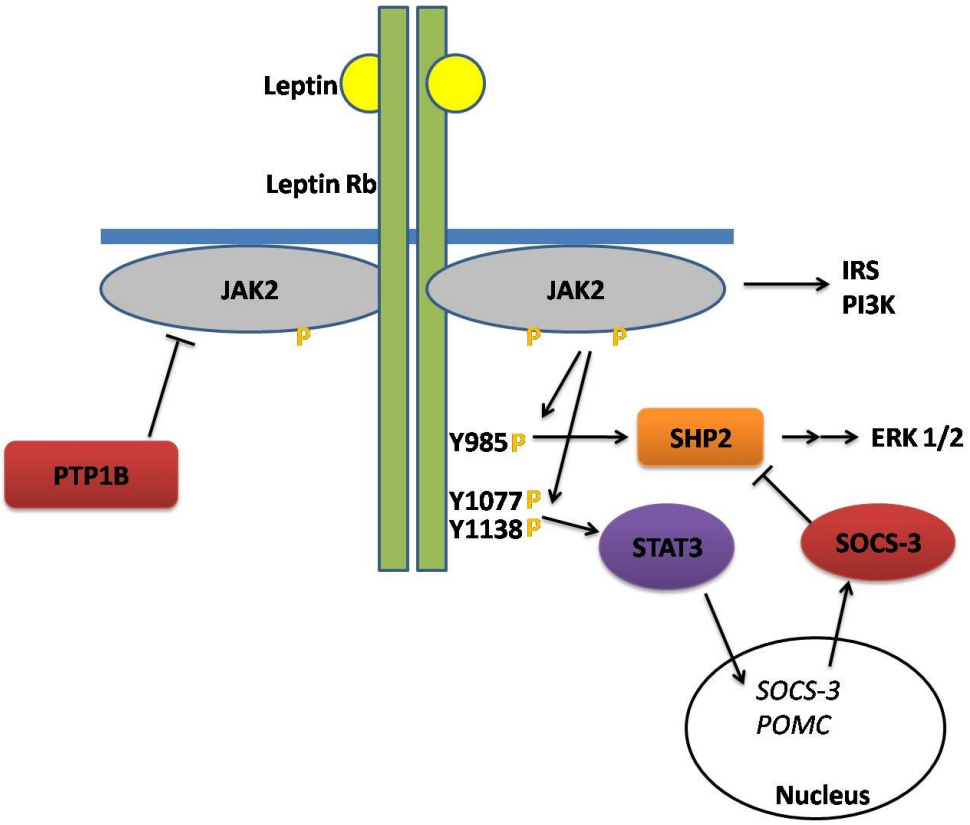

Figure 1.1 Leptin signaling involves multiple tyrosine phosphorylation events. Leptin binding results in activation and autophosphorylation of tyrosine kinase, JAK2. JAK2 can then phosphorylate the intracellular tail of LepRb including Y985, Y1077, and Y1138.

Through an elegant set of targeted substitution mutation studies, the metabolic contribution of each specific tyrosine site was determined by the Myers Lab at the University of Michigan. While Y985 phosphorylation leads to downstream ERK1/2, Y985 is also a binding site for a negative regulator of leptin signaling, suppressor of cytokine signaling-3 (SOCS-3) (Bjorbak et al., 2000). In mutant mice where Y985 is replaced with a leucine residue (l/l mice), normal STAT3 signaling persists, and l/l mice show a lean metabolic phenotype due to loss of SOCS-3 inhibition of leptin signaling (Björnholm et al., 2007). Replacement of Y1077 with phenylalanine residue (f/f mice) results in only a mild obese phenotype whereas serine substitution of Y1138 (s/s mice) results in a severe obese phenotype similar to *db/db* mice, suggesting that leptin-induced JAK2/STAT3 signaling mediates the majority of leptin's effects on energy balance (Bates et al., 2003; Patterson et al., 2012). In addition to phosphorylation of LepRb tyrosine residues,

JAK2 can phosphorylate insulin receptor substrate (IRS), allowing for downstream activation of the phosphoinositide-3 kinase (PI3K)-Akt signaling pathway (Duan et al., 2004; reviewed in Wauman and Tavernier, 2011).

Negative regulation of leptin signaling occurs via transcriptional feedback mechanisms as well as through phosphatase activity. SOCS-3 is a leptin-inducible protein that acts as a negative feedback inhibitor of leptin signaling via binding LepRb at Y985 (Fig. 1.1). Leptin injection in *ob/ob* mice induces hypothalamic SOCS-3 mRNA whereas leptin injection in *db/db* mice shows no effects (Bjørbaek et al., 1998). Furthermore, endogenous SOCS-3 expression is shown to be regulated by the fed state in rats (Baskin et al., 2000). In addition to negative transcriptional feedback, phosphatase activity reversing tyrosine phosphorylation plays a key role in leptin signaling termination. Numerous PTPs (PTP1B, RPTPe, SHP2, TCPTP, and PTEN) have been demonstrated to interact with the leptin signaling pathway, and increased PTP action has been implicated as a possible cause for cellular leptin resistance (St-Pierre and Tremblay, 2012; Tsou and Bence, 2012a). In diet-induced obesity mouse models, PTP1B and TCPTP expression are increased in the hypothalamus, suggesting PTPs may contribute to the pathogenesis of obesity (White et al., 2009; Loh et al., 2011).

Negative regulation of leptin signaling via phosphatase activity

The PTP superfamily consists of approximately 100 different PTPs, characterized by a highly conserved active site motif (I/V)HCXXGXXR(S/T) with a conserved cysteine residue which is essential for phosphate recognition and catalysis (Denu and Dixon, 1998; Tonks, 2003, 2006). Structural diversity within domains attached to the core recognition motif defines a PTP's substrate specificity and cellular localization. To date, five different PTPs have been demonstrated to regulate leptin signaling, acting upon different substrates along the pathway, and their roles in central control of metabolism have been studied through the use of genetic mouse models (Table 1.1). These include receptor-type protein tyrosine phosphatase epsilon (RPTPe),

Src homology 2 domain-containing protein tyrosine phosphatase (SHP2), T-cell protein tyrosine phosphatase (TCPTP), phosphatase and tensin homolog deleted on chromosome 10 (PTEN), and protein tyrosine phosphatase 1B (PTP1B) (St-Pierre and Tremblay, 2012).

RPTPe regulates leptin signaling via direct dephosphorylation of JAK2. Female RPTPe-deficient mice demonstrate resistance to diet-induced obesity and increased energy expenditure (Rousso-Noori et al., 2011). Interestingly, leptin-induced JAK2 activation results in phosphorylation of RPTPe on Y695 and enhances its ability to dephosphorylate JAK2, suggesting that central leptin signaling can be regulated by a phosphorylation-dependent negative feedback pathway (Rousso-Noori et al., 2011).

Early *in vitro* evidence suggested SHP2 could inhibit JAK2 activation or downregulate STAT3-mediated gene transcription, therefore acting as a negative regulator of leptin signaling (Carpenter et al., 1998; Li et al., 1999). More recently however, SHP2 has been shown to promote leptin signaling within the CNS, acting as an intermediate via binding Y985 of LepRb for eliciting downstream ERK1/2 signaling. SHP2's positive regulation of leptin signaling was further demonstrated through the development of CNS SHP2-/- mouse models. Forebrain/neuronal-specific or pan-neuronal SHP2-/- mice show an obese phenotype with increased weight gain and adiposity (Zhang et al., 2004; Krajewska et al., 2008). POMC neuron-specific deletion of SHP2 results in mice with increased body weight, adiposity, and serum leptin levels on both chow and HFD; POMC-SHP2-/- mice also display impaired leptin sensitivity and peripheral glucose homeostasis (Banno et al., 2010).

TCPTP negatively regulates leptin signaling via the direct dephosphorylation of STAT3. In a comprehensive study by Loh et al., neuronal TCPTP-/- mice were generated and metabolically phenotyped, providing *in vivo* evidence that TCPTP can regulate leptin-induced JAK/STAT signaling. Neuronal TCPTP-/- mice are resistant to diet-induced obesity and display

decreased adiposity and increased energy expenditure on HFD. Neuronal TCPTP-/- mice also show enhanced leptin sensitivity (increased leptin-induced pSTAT3 levels in hypothalamus and greater suppression of body weight/food intake) and peripheral glucose homeostasis as measured by glucose and insulin tolerance tests. Furthermore, Loh et al. generated neuronal PTP1B and TCPTP double knockouts; PTP1B-/-:TCPTP-/- mice are additively leaner and more leptin hyper-sensitive than single PTP1B-/- mice when fed a HFD, demonstrating an example of two PTPs acting on different substrates along the leptin pathway *in vivo* (Loh et al., 2011).

PTEN is a dual-specificity protein tyrosine phosphatase best known for its role as a tumor suppressor (Li et al., 1997; Steck et al., 1997). In spite of its role as a PTP, its primary physiological target appears to be the phospholipid PIP_3 rather than a tyrosine-phosphorylated protein (Maehama and Dixon, 1998). Thus, PTEN negatively regulates leptin signaling at the level of PI3K by directly dephosphorylating PIP_3 into PIP_2. Interestingly, the metabolic phenotypes of CNS PTEN mutant mouse models suggest a more complex role for PTEN in the central regulation of energy balance. LepRb-specific deletion of PTEN results in decreased body weight and adiposity, and oxygen consumption is increased compared to controls (Plum et al., 2007). Surprisingly, LepRb-specific overexpression (OE) of PTEN in mice does not result in an obese phenotype, and POMC- or ventromedial hypothalamus-specific PTEN deletion in mice leads to counterintuitive *increases* in body weight (Plum et al., 2006; Klöckener et al., 2011; Warne et al., 2011). Careful dissection of downstream PI3K pathways will be necessary in order to further understand PTEN's central metabolic role.

Perhaps the most well characterized PTP is PTP1B. PTP1B is a ubiquitously expressed phosphatase encoded by the human gene *PTPN1* (or *Ptpn1* in mouse) (Tonks et al., 1988a, 1988b; Charbonneau et al., 1989; Brown-Shimer et al., 1990). The human *PTPN1* gene is located on chromosome 20q13, a region which has been identified as a quantitative trait locus associated with obesity and type 2 diabetes (Lembertas et al., 1997; Ghosh et al., 1999; Lee et

al., 1999). More recently, a number of single nucleotide polymorphisms within the *PTPN1* gene associated with obesity and related metabolic disorders have been discovered in certain populations (Di Paola et al., 2002; Echwald et al., 2002; Kipfer-Coudreau et al., 2004; Palmer et al., 2004; Ukkola et al., 2005; Cheyssac et al., 2006; Meshkani et al., 2007; Bauer et al., 2010; Mo et al., 2010; Tsou and Bence, 2012b). As described below, PTP1B has been shown through both *in vitro* and *in vivo* studies to negatively regulate leptin and insulin signaling, functionally demonstrating its role in the central regulation of energy balance and glucose homeostasis.

PTP1B and the metabolic effects of PTP1B deficiency

PTP1B was first isolated from human placenta as a 37kDa protein, but further isolation of a cDNA clone for human PTP1B found the full length protein to be ~50kDa (Tonks et al., 1988a, 1988b; Brown-Shimer et al., 1990; Chernoff et al., 1990). It was the first PTP to be purified and to have its crystal structure determined (Tonks et al., 1988a; Barford et al., 1994). The C-terminus of PTP1B plays a role in its subcellular localization; the hydrophobic C-terminal 35 residues target the enzyme to the cytoplasmic face of the endoplasmic reticulum (Frangioni et al., 1992). Proteolytic cleavage by calpain of the C-terminus generates a soluble form of PTP1B with increased activity (Frangioni et al., 1993). Early *in vitro* studies established PTP1B as an insulin receptor phosphatase. Purified PTP1B was shown to antagonize insulin signaling when injected into *Xenopus* oocytes. PTP1B injection increased endogenous levels of PTPase activity and blocked insulin-stimulated tyrosine-phosphorylation (Cicirelli et al., 1990). Through the use of site-directed mutagenesis, catalytically inactive “substrate-trapping” mutants were generated in order to identify PTP1B targets (Flint et al., 1997). Substrate-trapping findings combined with structural evidence led to the determination that PTP1B preferentially binds to the tandem phospho-tyrosine motif (E/D)-pY-pY-(R/K), due to the presence of an additional phospho-tyrosine recognition site adjacent to its catalytic site. PTP1B was shown to preferentially bind and dephosphorylate the insulin receptor (IR) at pY1162/pY1163 (Salmeen et al., 2000) and the tyrosine kinases JAK2 and TYK2 at pY1007/pY1008 and pY1054/pY1055, respectively (Myers et

al., 2001). Furthermore, PTP1B has been shown to dephosphorylate other receptor tyrosine kinases including epidermal growth factor receptor (Flint et al., 1997), platelet-derived growth factor receptor (Liu and Chernoff, 1997), and insulin-like growth factor receptor 1 (Kenner et al., 1996). It is also likely that PTP1B can dephosphorylate tyrosine phosphorylated proteins such as IRS-1 (Goldstein et al., 2000), Src (Bjorge et al., 2000), and p62Dok (Dubé et al., 2004).

PTP1B's role in energy homeostasis was first revealed by the generation of PTP1B-deficient mice. By targeting deletion of exons 5 and 6 of the *Ptpn1* gene, Elchebly et al. generated global PTP1B-/- mice which were shown to be insulin hyper-sensitive and resistant to high-fat diet induced obesity when compared to PTP1B+/+ animals. PTP1B-/- mice show enhanced and/or prolonged insulin-stimulated insulin receptor phosphorylation in liver and muscle (Elchebly et al., 1999). A separate line of PTP1B-/- mice was generated by disrupting the ATG-coding exon 1 by Klaman et al in 2000. These mice were thoroughly metabolically phenotyped, and consistent with the findings from Elchebly et al., this line of PTP1B-/- mice also showed decreased body weight on HFD and improved peripheral glucose homoeostasis. Furthermore, Klaman et al. determined that PTP1B-/- mice have increased energy expenditure, providing a likely explanation for the lean metabolic phenotype. PTP1B-/- mice also demonstrate decreased serum leptin levels, suggesting that they might be leptin hypersensitive (Klaman et al., 2000). Indeed, PTP1B-deficiency was later shown to improve leptin sensitivity both *in vitro* and *in vivo* (Zabolotny and Bence et al., 2002; Cheng et al., 2002). In 2002, Zabolotny and Bence et al. and Cheng et al. found evidence that PTP1B negatively regulates leptin signaling. Zabolotny and Bence et al. showed that leptin-induced phosphorylation of JAK2 and STAT3 is reduced in COS-7 cells coexpressing LepR and PTP1B when compared to cells expressing LepR alone (Zabolotny et al., 2002). Both groups found increased leptin sensitivity in PTP1B-/- animals compared to WT controls. Using PTP1B substrate-trapping mutants, both groups determined that PTP1B's regulatory effects on leptin signaling occurs via direct dephosphorylation of JAK2 (Flint et al., 1997; Cheng et al., 2002; Zabolotny et al., 2002).

Through the use of conditional mouse genetics, a number of tissue-specific PTP1B-deficient mouse models have been generated, providing insight into PTP1B's central and peripheral metabolic effects. Brain-specific PTP1B-/- mice (generated with a nestin-cre transgenic line) recapitulate the whole body PTP1B-/- phenotype and show improved body weight and adiposity compared to controls on both chow and HFD; these mice display both decreased food intake and increased energy expenditure, and are leptin and insulin hypersensitive (Bence et al., 2006). Interestingly, weight-matched female neuron-specific PTP1B-/- mice continue to demonstrate improved insulin sensitivity compared to controls, suggesting that central PTP1B deficiency may affect peripheral glucose homeostasis independent of body weight differences (Bence et al., 2006). Mice deficient in PTP1B specifically within POMC neurons (POMC-PTP1B-/-) also show decreased body weight and adiposity, albeit only when fed a high-fat diet, due to increased energy expenditure. Notably, on a chow diet POMC-PTP1B-/- mice show improved glucose tolerance and insulin sensitivity even when controlled for body weight and adiposity, confirming that central PTP1B can regulate peripheral glucose homeostasis (Banno et al., 2010). POMC-PTP1B-/- mice also display enhanced leptin sensitivity within the hindbrain and a heightened homeostatic response to cold exposure (De Jonghe et al., 2011a, 2012).

In contrast to CNS-PTP1B deficiency, peripheral PTP1B deletion does not yield a lean body weight phenotype. Liver- and muscle-specific PTP1B deletion results in improvements in peripheral glucose homeostasis and enhanced insulin sensitivity without effects on body weight or adiposity (Bence et al., 2006; Delibegovic et al., 2007, 2009). Notably, liver-specific PTP1B-/- mice also exhibit decreased markers of endoplasmic reticulum (ER) stress on a HFD (Delibegovic et al., 2009; Agouni et al., 2011). The role of PTP1B in adipose is less clear; using the aP2-driven Cre to generate PTP1B deficiency in fat tissue results in mice with *increased* body weight on HFD. However, whether adipocyte-specific PTP1B deletion explains the increased body weight phenotype of aP2-PTP1B-/- mice is unclear. Only a ~50% reduction in PTP1B expression

was observed in adipocytes isolated from WAT of aP2-PTP1B-/- mice, and aP2-Cre-mediated recombination has been shown to occur in other cell types including macrophages, osteoblasts, and cardiomyocytes (Bence et al., 2006; Mao et al., 2009; Wang et al., 2010). More recently, adipocyte-specific PTP1B-/- mice were generated using the adiponectin-Cre line in order to achieve a more efficient, adipocyte-specific deletion (Owen et al., 2012). Adipocyte-specific PTP1B deletion has no effect on body weight, glucose homeostasis, or total adiposity, but increases adipocyte size, circulating glucose and leptin levels (Owen et al., 2012). Macrophage-specific PTP1B deletion has no effect on body weight but improves peripheral glucose and insulin tolerance on HFD (Grant et al., 2013), suggesting the increased body weight observed in aP2-PTP1B-/- mice is not attributed to adipocyte nor macrophage PTP1B loss. Taken together, the findings from the tissue-specific PTP1B-/- models indicate that central PTP1B-deficiency decreases body weight/adiposity and improves peripheral glucose homeostasis, while PTP1B-deficiency in muscle or liver does not alter body weight but does significantly improve insulin sensitivity and glucose tolerance.

Non-leptin signaling pathways implicated in the central control of energy homeostasis

Despite strong evidence that PTP1B centrally regulates energy balance via its interaction with leptin signaling, whether or not the metabolic effects of PTP1B deficiency are exclusively due to enhanced leptin signaling remains unknown. Indeed, there is evidence for PTP1B's leptin independent metabolic effects. In 2002, Cheng et al. found that leptin and PTP1B double mutants (*ob/ob*;PTP1B-/-) show a leaner phenotype and increased resting metabolic rate (normalized to body weight) than single leptin-deficient *ob/ob* mice, suggesting that in addition to regulation of leptin signaling, PTP1B may also have leptin-*independent* effects important in maintaining energy homeostasis (Cheng et al., 2002). Since *ob/ob*:PTP1B-/- mice continue to express leptin receptors, the leaner phenotype of the double mutants compared to *ob/ob* single mutants could also possibly be explained by ligand-independent activation and sensitized basal activity of LepRb. Thus, whether or not PTP1B can regulate non-leptin pathways implicated in

the CNS control of energy balance is unclear. Furthermore, other non-leptin signaling pathways have been implicated in the central control of energy homeostasis including insulin and non-leptin cytokines such as interleukin-6 (IL-6) or ciliary neurotrophic factor (CNTF), and there is evidence that these signaling pathways are regulated by PTP1B as outlined below (Fig. 1.2).

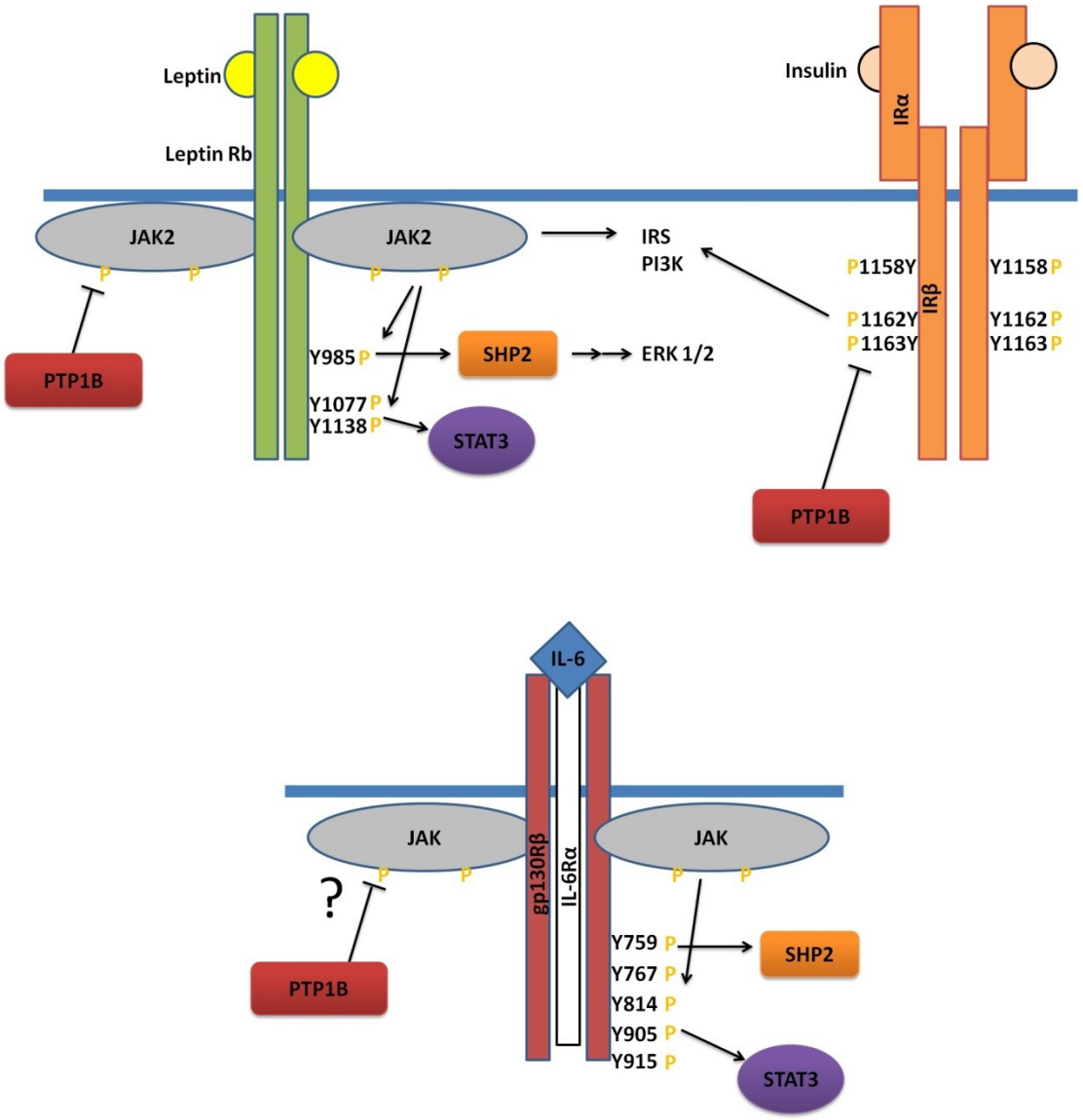

Figure 1.2 PTP1B can regulate non-leptin pathways. In addition to regulation of leptin signaling, PTP1B is a known regulator of insulin signaling and a potential regulator of non-leptin cytokine signaling such as IL-6.

Insulin is a pancreas-secreted hormone classically studied for its ability to stimulate uptake of glucose and nutrients into peripheral target tissues. In the CNS however, insulin, like leptin, has been shown to suppress body weight and food intake (Woods et al., 1979; Air et al., 2002a) and affect neuropeptide gene expression and hypothalamic neuron activity (Schwartz et al., 1992; Benoit et al., 2002; Choudhury et al., 2005). Similar to leptin signaling, insulin signaling relies on phosphotyrosyl residues to elicit its downstream effects. In contrast to LepRb however, the IR has intrinsic tyrosine kinase activity; upon ligand binding, the transmembrane β subunits of the IR autophosphorylate on tyrosines Y1158, Y1162, and Y1163, recruiting downstream effector proteins including IRS. IRS becomes phosphorylated by IR on various Y residues and can activate a number of downstream signaling pathways including the phosphoinositide-3 kinase (PI3K)-Akt and MAPK pathways (reviewed in White, 1997). Historically, much of what is known regarding the cellular mechanisms underlying insulin signaling has been studied within the periphery, however, there are numerous studies supporting a connection between CNS insulin signaling and energy homeostasis. Neuron-specific IR knockout (NIRKO) mice demonstrated genetic evidence that central IR signaling plays an important role in regulating body weight (Brüning et al., 2000). NIRKO mice displayed a mild obesity phenotype with increased body weight and adiposity on HFD and mild insulin resistance as measured by insulin tolerance test (Brüning et al., 2000). Furthermore, pharmacological studies have revealed that the reduction of food intake by acute central insulin administration is PI3K-dependent (Niswender et al., 2003), and *in vitro* models have identified the MAPK pathway as important for insulin's ability to regulate NPY/AgRP gene expression (Mayer and Belsham, 2009). PTP1B has been historically linked to regulating insulin signaling in the periphery, and less is known regarding its interaction with central insulin signaling. In 2008, Picardi et al. demonstrated that hypothalamic PTP1B can regulate insulin signaling and insulin-induced food intake suppression. Using anti-sense oligonucleotides, Picardi et al. knocked down PTP1B expression within the hypothalamus of diet-induced obese rats and delivered insulin and measured food intake suppression. Rats with hypothalamic PTP1B knockdown demonstrated enhanced insulin-induced food intake

suppression. Insulin-induced IR or IRS phosphorylation was also enhanced in the hypothalamus of rats with PTP1B knockdown (Picardi et al., 2008).

In addition to leptin, other cytokine signaling pathways have been shown to play a role in the control of energy homeostasis. Obesity is associated with a state of chronic low-level inflammation as macrophage accumulation and increased levels of pro-inflammatory cytokines are detected within adipose tissue (Wellen and Hotamisligil, 2005). IL-6 and CNTF are part of the IL-6-type cytokine family, most studied for their role in inflammation and immune function. IL-6 mediates a variety of physiological functions including induction of acute phase proteins, cell proliferation, survival, and apoptosis (Hodge et al., 2005). CNTF is also involved in numerous cellular functions including cell survival, differentiation, and neuroprotection after acute injury (Sleeman et al., 2000). Like leptin, IL-6-type cytokines signal through type I cytokine receptors. Unlike leptin however, the receptors are complexes containing α and β subunits. The α-subunits are non-signaling receptors which bind the cytokine ligand. They complex with a dimer of signaling β subunits that includes at least one copy of the transmembrane signal transduction protein gp130Rβ (Febbraio, 2007). The gp130Rβ shares sequence homology with LepRb, and both activate similar downstream signaling pathways such as JAK/STAT and ERK signaling pathways (Ernst and Jenkins, 2004). IL-6 signals through a gp130Rβ homodimer whereas CNTF signals through a heterodimer of gp130Rβ and LIFRβ receptors (Heinrich et al., 2003). When IL-6 binds the IL-6Rα, gp130Rβ homodimerization occurs, allowing for signaling via activation of associated tyrosine kinases JAK1, JAK2, and TYK2. CNTF can likewise activate gp130. The gp130 associated janus kinases autophosphorylate and phosphorylate residues of the cytoplasmic tail of gp130Rβ, allowing for binding of STAT transcription factors such as STAT3 and STAT1 (Heinrich et al., 1998). Similar to transcriptional regulation of LepRb signaling, STAT3-dependent transcription of SOCS provides negative regulation of IL-6-type receptor signaling (Heinrich et al., 2003). Additional negative regulation of IL-6-type cytokine signaling likely occurs via receptor endocytosis as well as JAK/STAT dephosphorylation, suggesting a

possible role for PTP1B as a negative regulator of IL-6-type cytokine signaling (Castell et al., 1988; Heinrich et al., 1998, 2003; Chang, 2011).

IL-6-type cytokines have been implicated in the central control of energy homeostasis. A large proportion of circulating IL-6 is secreted from adipocytes and, like leptin, blood levels correlate with adipose tissue mass (Mohamed-Ali et al., 1997; Bastard et al., 2000). Whether or not circulating IL-6 can cross the blood brain barrier intact and affect the CNS is incompletely understood (Banks et al., 1994). However, IL-6 has also been shown to be expressed within the CNS in hypothalamic nuclei important for energy homeostasis (Schöbitz et al., 1993; Shizuya et al., 1998). Wallenius et al. showed that mice lacking IL-6 develop mature-onset obesity. Mature IL6-/- animals additionally showed increased circulating leptin levels and leptin resistance. Interestingly, low dose IL-6 replacement reduced body weight and leptin levels in IL6-/- animals (Wallenius et al., 2002b). The same group also went on to show that IL-6 delivered ICV in rats can affect energy expenditure and body weight. Single ICV injection of IL-6 increased oxygen consumption and carbon dioxide production in male rats, while chronic daily ICV injections suppressed body weight and decreased total fat pad mass (Wallenius et al., 2002a, 2002b).

CNTF has also been shown to have central effects on body weight. For example, peripheral CNTF injections have weight reducing effects in *ob/ob*, diet-induced obese mice resistant to leptin, and even in *db/db* mice (Gloaguen et al., 1997; Lambert et al., 2001). Furthermore, CNTF administration can induce hypothalamic STAT3 phosphorylation (Lambert et al., 2001). Direct central administration of CNTF reduces body weight and is blocked by the orexigenic effects of NPY coinfusion (Pu et al., 2000). Interestingly, CNTF's food intake and weight reducing effects do not appear to be localized to LepRb-expressing neurons, even though CNTF signaling in POMC neurons appears to play a role (Janoschek et al., 2006; Stefater et al., 2012a). Subcutaneous CNTF delivery also continues to have weight reducing effects in mice with mediobasal hypothalamus lesions (Anderson et al., 2003).

Though there is limited evidence implicating PTP1B in the regulation of IL-6-type cytokine signaling, a few studies have linked PTP1B to IL-6. In smooth muscle cell cultures, IL-6 induced JAK/STAT signaling is enhanced with PTP1B knockdown (Chang, 2011). Additionally, IL-6 has been shown to downregulate hypothalamic PTP1B protein expression, improving central leptin and insulin action (Chiarreotto-Ropelle et al., 2013). One study has examined CNTF signaling in relation to PTP1B; in a human neuroblastoma cell line, CNTF treatment does not induce PTP1B expression, nor does PTP1B overexpression affect CNTF-induced JAK2 and STAT3 activation (Benomar et al., 2009). However, given that PTP1B's substrates include JAK2 and TYK2 (Myers et al., 2001), two janus kinases associated with gp130 receptor signaling, PTP1B may be a regulator of IL-6 and/or CNTF downstream gp130 activation.

Taken together, there is mounting evidence of potential PTP1B-regulated, non-leptin signaling pathways which play a role in the central control of energy balance. We hypothesize that PTP1B's contribution to the central regulation of energy homeostasis occurs via both leptin-dependent and -independent pathways. This dissertation examines the role of PTP1B in leptin-dependent and -independent pathways through a comprehensive metabolic study of novel genetic mouse models as well as an *in vitro* signaling study.

To examine whether PTP1B can regulate non-leptin signaling pathways involved in the CNS control of energy balance, we first began with an indirect approach, examining whether or not LepRb-specific PTP1B deficiency demonstrates a partial or full recapitulation of whole body PTP1B deficiency. As detailed in chapter 2, we generated LepRb-specific PTP1B-/- mice and compared them to global PTP1B-/- mice in a battery of metabolic phenotyping measures. As discussed in chapter 3, we next generated mice lacking both PTP1B and LepRb in the hypothalamus (Nkx2.1-PTP1B-/-:LepRb-/- mice) and compared them to hypothalamus-targeted LepRb-/- mice (Nkx2.1-LepRb-/- mice) to determine whether PTP1B deficiency can sensitize non-leptin pathways and improve the obese phenotype caused by genetic LepRb deletion in the

hypothalamus. Chapter 4 details our use of an immortalized mouse hypothalamic cell line to study the effects of PTP1B knockdown or overexpression on IL-6 induced JAK/STAT signaling. Finally, in chapter 5 we discuss our findings within the context of the field and propose future directions for additional study.

PTP mouse model	Body Weight and Adiposity Phenotype	Leptin sensitivity	Glucose homeostasis	References
Global RPTPe-/- (whole body)	↑ in females	↑ in females	Improved GTT Improved ITT	Rousso-Noori et al. 2011
Forebrain-specific SHP2-/- (CaMKIIα-Cre)	↑	↓	(Fed) Hyperglycemia Hyperinsulinemia Fatty liver	Zhang et al. 2004
Brain-specific SHP2-/- (CRE3)	↑	↓	Impaired glucose tolerance Hyperinsulinemia	Krajewska et al. 2005
POMC-neuron specific SHP2-/- (POMC-Cre)	↑	↓	Hyperinsulinemia Impaired glucose tolerance	Banno et al. 2010
Neuron-specific TCPTP-/- (Nestin-Cre)	↓	↑	Improved GTT Improved ITT	Loh et al. 2011
Neuron-specific TCPTP-/- :PTP1B-/- (Nestin-Cre)	↓ (additive effects)	↑ (additive effects)	Improved ITT (additive effects)	Loh et al. 2011
LepRb-specific PTEN-/- (LepRb-Cre)	↓	Increased leptin-induced PI3K pathway	Improved GTT Improved ITT Decreased serum insulin	Plum et al. 2007
LepRb-specific PTEN overexpression (LepRb-Cre)	No change	Decreased leptin-induced PI3K pathway	Fatty liver	Warne et al. 2011
POMC-PTEN-/- (POMC-Cre)	↑, sex and diet-dependent	Decreased food intake suppression	ND	Plum et al. 2006
VMH-specific PTEN-/- (SF1-Cre)	↑	ND	ND	Klöckener et al., 2011
Global PTP1B -/- (whole body)	↓	↑	Improved GTT Improved ITT	Klaman et al. 2000, Echelby et al. 1999
Neuron-specific PTP1B -/- (Nestin-Cre)	↓	↑	Improved GTT Improved ITT	Bence et al. 2006
POMC-neuron specific PTP1B -/- (POMC-Cre)	↓	↑	Improved GTT Improved ITT	Banno et al. 2010

Table 1.1 Summary of CNS PTP-genetic mouse models and their associated metabolic phenotypes. ↑, increased. ↓, decreased. ND, not determined.

CHAPTER 2: Deficiency of PTP1B in LepRb-Expressing Neurons Leads to Decreased Body Weight and Adiposity in Mice

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Abstract

Protein tyrosine phosphatase 1B (PTP1B) is a ubiquitously expressed tyrosine phosphatase implicated in the negative regulation of leptin and insulin receptor signaling. PTP1B$^{-/-}$ mice possess a lean metabolic phenotype attributed at least partially to improved hypothalamic leptin sensitivity. Interestingly, mice lacking both leptin and PTP1B (*ob/ob*:PTP1B$^{-/-}$) have reduced body weight compared to mice lacking leptin only, suggesting that PTP1B may have important leptin-*independent* metabolic effects. We generated mice with PTP1B-deficiency specifically in leptin receptor (LepRb) expressing neurons (LepRb-PTP1B$^{-/-}$) and compared them to LepRb-Cre only wild type controls (WT) and global PTP1B$^{-/-}$ mice. Consistent with PTP1B's role as a negative regulator of leptin signaling, our results show that LepRb-PTP1B$^{-/-}$ mice are leptin hypersensitive and have significantly reduced body weight when maintained on chow or high-fat diet (HFD) compared to WT controls. LepRb-PTP1B$^{-/-}$ mice have a significant decrease in adiposity on HFD compared to controls. Notably, the extent of attenuated body weight gain on HFD, as well as the extent of leptin hypersensitivity, is similar between LepRb-PTP1B$^{-/-}$ mice and global PTP1B$^{-/-}$ mice. Overall, these results demonstrate that PTP1B-deficiency in LepRb-expressing neurons results in reduced body weight and adiposity compared to WT controls and likely underlies the improved metabolic phenotype of global and brain-specific PTP1B-deficient models. Subtle phenotypic differences between LepRb-PTP1B$^{-/-}$ and global PTP1B$^{-/-}$ mice,

however, suggest that PTP1B independent of leptin signaling may also contribute to energy balance in mice.

Introduction

Obesity is a growing health problem in the United States and worldwide (Ahima, 2006; Flegal et al., 2012). Numerous cellular signals play a role in body weight regulation including leptin. Leptin is a 16 kDa hormone released by adipose tissue into circulation which plays a major regulatory role in feeding and energy expenditure via action in the brain (Zhang et al., 1994). Circulating leptin levels correlate with total body fat content, signaling to the central nervous system (CNS) current levels of energy stores. Increasing levels of leptin signal a state of energy abundance to the brain, suppressing food intake and increasing energy expenditure. Leptin-deficient *ob/ob* mice are hyperphagic and develop obesity and insulin resistance (Pelleymounter et al., 1995).

The leptin receptor has multiple isoforms and only the "long form," LepRb, is capable of associating with the intracellular tyrosine kinase Janus kinase 2 (JAK2). Leptin stimulation activates JAK2 resulting in autophosphorylation and phosphorylation of several tyrosine residues on LepRb allowing for recruitment and activation of downstream signaling molecules. LepRb has broad central expression within the forebrain (hippocampus, hypothalamus, piriform cortex), midbrain (dorsal raphe nuclei), and hindbrain (cerebellum, reticulotegmental nucleus, nucleus of the solitary tract; NTS) (Elmquist et al., 1998; Leshan et al., 2006; Patterson et al., 2011). The highest concentrations of LepRb-expressing neurons are found in hypothalamic nuclei including the arcuate nucleus, ventromedial and dorsomedial nuclei of the hypothalamus, and the lateral hypothalamus. Within the arcuate nucleus, two separate neuron populations expressing either agouti-related protein (AgRP) or proopiomelanocortin (POMC) drive opposing effects on energy balance. Leptin decreases food intake and increases energy expenditure by inhibiting orexigenic AgRP neuron activity while stimulating anorexigenic POMC neurons. In the hindbrain, leptin

induces pSTAT3 immunoreactivity in POMC NTS neurons and is also required for energy balance regulation (Ellacott et al., 2006; Hayes et al., 2010).

Leptin signaling is regulated by multiple mechanisms including tyrosine phosphorylation. Protein tyrosine phosphatase 1B (PTP1B) is an important negative regulator of leptin signaling through the dephosphorylation of JAK2 (Cheng et al., 2002; Zabolotny et al., 2002). PTP1B is a ubiquitously expressed enzyme, however in the brain it shows enriched expression in areas of high LepRb expression, including in the hypothalamus (Zabolotny et al., 2002). Consistent with its role as a negative regulator of leptin signaling, global-, whole brain-, or POMC neuron-specific deficiency of PTP1B in mice results in lean and leptin hyper-sensitive animals (Klaman et al., 2000; Bence et al., 2006; Banno et al., 2010). Muscle-, liver-, or adipocyte-specific PTP1B deletion does not result in a reduction in body weight or adiposity, therefore PTP1B's regulation of body mass is likely to be centrally mediated (Bence et al., 2006; Delibegovic et al., 2007, 2009; Owen et al., 2012). Global PTP1B knockout mice also show improvements in insulin sensitivity, consistent with PTP1B being a known negative regulator of insulin receptor signaling (Ahmad et al., 1995; Kenner et al., 1996). Indeed, deficiency of PTP1B in major insulin responsive tissues (muscle or liver) results in enhanced insulin receptor signaling and improved glucose homeostasis (Delibegovic et al., 2007, 2009). Interestingly, neuron-specific and POMC-neuron specific PTP1B knockout mice also show improvements in peripheral insulin sensitivity even when normalized for body weight and adiposity (Bence et al., 2006; Banno et al., 2010).

The lean metabolic phenotype of PTP1B-deficient mouse models is primarily attributed to improved leptin sensitivity; thus, compound *ob/ob*:PTP1B$^{-/-}$ mice would be expected to exhibit a similar metabolic phenotype compared to *ob/ob* single mutants, due to the complete loss of circulating leptin. Intriguingly however, *ob/ob*:PTP1B$^{-/-}$ mice exhibit suppressed body weight gain in comparison to *ob/ob* single mutants, suggesting that PTP1B's metabolic effects may not be solely due to its regulation of leptin signaling (Cheng et al., 2002). In a separate study, *ob/ob*

mice treated with PTP1B anti-sense oligonucleotides showed decreased epididymal fat pad weight when compared to saline treated mice (Zinker et al., 2002). Additionally, leptin receptor-deficient PTP1B double mutants (*db/db*:PTP1B$^{-/-}$) exhibit decreased plasma triglyceride and free fatty acids compared to *db/db*:PTP1B$^{+/-}$ mice (Ali et al., 2009). Thus, the extent to which PTP1B mediates its metabolic effects via central leptin-independent pathways remains unclear. Here, to begin to address this, we generated mice with PTP1B-deficiency in leptin receptor-expressing neurons (LepRb-PTP1B$^{-/-}$) and examined body weight, adiposity, leptin sensitivity and glucose homeostasis. LepRb-PTP1B$^{-/-}$ and LepRb-PTP1B$^{+/-}$ mice were compared to LepRb-Cre controls as well as to whole-body PTP1B knockouts to examine whether PTP1B's regulation of energy homeostasis is limited to leptin receptor-expressing neurons.

Materials and Methods

Animal care. All animal care protocols and procedures were approved by the University of Pennsylvania Institutional Care and Use Committee. We maintained mice on a 12-hour light/12-hour dark cycle in a temperature- controlled barrier facility, with free access to water and food (standard chow autoclavable Lab Diet 5010 or custom HFD Teklad TD93075). Age-matched littermates were used for all experiments.

Mice with Lepr-specific deletion of PTP1B. *Ptpn1*$^{loxP/loxP}$ mice were generated previously and genotyped by PCR as described (Bence et al., 2006). Genotyping primer sequences for the Ptpn1 allele were: PTP1B forward 5′-TGCTCACTCACCCTGCTACAA, PTP1B reverse 5′-GAAATGGCTCACTCCTACTGG; All *Ptpn1*$^{loxP/loxP}$ mice were originally on a mixed 129Sv/J × C57BL/6 background prior to mating with *LepRb-Ires-Cre* mice (gift from M. Meyers, University of Michigan, Ann Arbor, Michigan), which were on a C57BL/6 background. Genotyping primer sequences for *LepRb-Ires-Cre* allele were:

Cre forward 5'-CCTCTCCACCCAAGCGGCCGGAGAACC

Cre reverse 5'-CCGGCTCCGTTCTTTGGTGGCCCCTTCGCG

WT forward 5'-GCCCTCATTAATCTAGTAATGTAGATGG

WT reverse 5'-ACTAGGGGTCAACTCTC

Isolating DNA from tissues for detection of recombination of the floxed alleles. Tissues were digested at 55°C overnight in proteinase K digestion buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 300 µg/ml proteinase K). Saturated NaCl (~6M) was added to the digestion, and samples were vortexed vigorously for 1 minute. Samples were centrifuged for 20 minutes at 13,700 g, and supernatants were transferred to a fresh tube. DNA was precipitated by adding 1 ml 100% ethanol, and pellets were washed once with 70% ethanol and were resuspended in 100 µl of sterile PCR water for analysis. PCR primers for detection of recombined alleles were: *Ptpn1*Δ/Δ forward 5′-GTGGTGCCTGCAAGAGAACTGAC

*Ptpn1*Δ/Δ reverse 5′-GAAATGGCTCACTCCTACTGG

IL-2 internal control forward 5′-CTAGGCCACAGAATTGAAAGATCT

IL-2 internal control reverse 5′-GTAGGTGGAAATTCTAGCATCATCC

Body composition and food intake. At weaning, mice were placed on diets of either standard laboratory chow (Lab Diet 5010; calories provided by protein [28.7%], fat [12.7%], and carbohydrate [58.5%]) or custom HFD (Teklad TD93075; calories provided by protein [21.2%], fat [54.8%], and carbohydrate [24%]). Body weights were assessed weekly for at least 18 weeks and food intake was measured daily. Body length was measured as nose-rump length at indicated age. Epididymal fat pads were dissected and weighed at indicated age.

Energy expenditure measurements. Adult male mice were transitioned from chow diet to HFD at 9 weeks of age. Rectal temperature was measured with a thermistor during light cycle in animals at 11 weeks of age (MicroTherma 2T; ThermoWorks). Brown adipose tissue UCP1 gene expression was measured by qRT-PCR as described below.

Leptin sensitivity. For *in vivo* leptin sensitivity measurements, recombinant mouse leptin 0.5 µg/g BW/injection (A.F. Parlow, NHPP) or an equal volume 0.9% saline was administered i.p. to male mice on a chow diet (~7–8 weeks of age). Mice were initially injected with saline i.p. every 12 hours over the course of 36 hours. After a recovery period of 24 hours, leptin was administered morning and evening following the same paradigm. Body weight and food intake were monitored daily for 4 days following the initial leptin injection. Baseline body weight and food intake measurements for the days prior to the start of leptin injections were averaged and used to calculate percent change. Animals acted as internal controls as each received both saline and leptin injections using a within subjects design.

Glucose homeostasis. Glucose tolerance tests (GTTs) were performed as described previously (Klaman et al., 2000). Glucose dose used for i.p. injections was 2 mg/g BW (20% solution). Blood glucose was assayed in tail blood using a glucometer (Contour, Bayer). Fasting insulin levels were determined as described below.

RNA extraction and real-time PCR. Mice were sacrificed at the onset of the light cycle (08:00-10:00 a.m.) after overnight food withdrawal (fasted). Tissues were rapidly dissected and flash frozen in liquid nitrogen. Total RNA was extracted from tissues using TRIzol (Invitrogen) and the RNeasy kit (QIAGEN). cDNA was synthesized from 1 µg total RNA using the Advantage RT-for-PCR kit (Clontech). The relative mRNA levels of UCP1, and Cidea were assessed and quantified by quantitative real-time PCR (qRT-PCR). HPRT-1 (SABiosciences) was used as an internal control. The qRT-PCR reactions were carried out using RT² SYBR Green qPCR Master Mix (SABiosciences), and samples were run using the Eppendorf Mastercycler ep realplex. Primer sequences for UCP1 and Cidea were reported previously (Seale et al., 2007). Relative mRNA expression was calculated using the comparative Ct method as described previously (Bence et al., 2006).

Serum analysis. Trunk blood was collected from mice after overnight fast at indicated age. Serum was separated by centrifugation at 6,000 g. Serum insulin and leptin were measured by ELISA(CrystalChem).

Statistics. Results are expressed as mean ± SEM. Comparisons between groups were made by unpaired 2-tailed Student's t test or 2-way ANOVA with repeated measures in one factor followed by Fisher's protected least significant difference (PLSD) test, as appropriate. A p value less than 0.05 was considered to be statistically significant.

Results

Generation of LepRb-specific PTP1B–/– mice.

To generate LepRb-specific PTP1B-deficient mice, we crossed *Ptpn1*$^{loxP/loxP}$ mice to *LepRb*$^{cre/cre}$ knock-in mice in which Cre recombinase is specifically expressed in LepRb-expressing-cells (*LepRb-Ires-Cre*) (Leshan et al., 2006, 2009; Piper et al., 2008; Xu et al., 2012). The resulting *Ptpn1*$^{+/loxP}$ *LepRb*$^{+/cre}$ mice were subsequently crossed to *LepRb*$^{cre/cre}$ mice to yield *Ptpn1*$^{+/loxP}$ *LepRb*$^{cre/cre}$ mice. *Ptpn1*$^{+/loxP}$ *LepRb*$^{cre/cre}$ mice were crossed together to yield *Ptpn1*$^{loxP/loxP}$ *LepRb*$^{cre/cre}$ (hereafter termed LepRb-PTP1B$^{–/–}$), *Ptpn1*$^{+/loxP}$ *LepRb*$^{cre/cre}$ (hereafter termed LepRb-PTP1B$^{+/–}$), and *Ptpn1*$^{+/+}$ *LepRb*$^{cre/cre}$ (LepRb-Cre) "wild type," Cre-only littermate controls. In order to increase numbers of *Ptpn1*$^{loxP/loxP}$ *LepRb*$^{cre/cre}$ and to generate all appropriate controls, we also crossed *Ptpn1*$^{+/loxP}$ *LepRb*$^{+/cre}$ mice with *Ptpn1*$^{loxP/loxP}$ mice to yield *Ptpn1*$^{loxP/loxP}$ *LepRb*$^{+/cre}$ which were then subsequently crossed together. This generated additional *Ptpn1*$^{loxP/loxP}$ *LepRb*$^{cre/cre}$ (LepRb-PTP1B$^{–/–}$) and *Ptpn1*$^{loxP/loxP}$ *LepRb*$^{+/+}$ (PTP1B fl/fl) controls. Finally, in a parallel cross we crossed PTP1B$^{+/–}$ mice together to generate whole-body PTP1B$^{–/–}$ and their respective PTP1B $^{+/+}$ "wild type" littermate controls.

To verify deletion of the PTP1B gene, we extracted DNA from a variety of tissues (hypothalamus, brain without hypothalamus, pituitary, liver, hindlimb muscle, epididymal white adipose,

interscapular brown adipose, lung, heart, and kidney) and assessed for deletion of the floxed allele via PCR. Deletion was only detected in hypothalamus tissue from LepRb-PTP1B$^{-/-}$ (Fig. 2.1). Although LepRb is also expressed outside of the hypothalamus (Elmquist et al., 1998; Mercer et al., 1998; Leshan et al., 2006), no deletion was detected in whole brain without hypothalamus or peripheral tissues of LepRb-PTP1B$^{-/-}$ mice, likely due to dilution of signal by non-LepRb expressing cells and generally limited LepRb expression. No deletion was detected in any tissues isolated from wild type control animals while deletion of the PTP1B gene was detected in all tissues from whole-body PTP1B$^{-/-}$ mice acting as positive controls.

LepRb-PTP1B$^{-/-}$ mice have decreased body weight on chow and high-fat diet.

To assess whether energy balance is affected by PTP1B-deficiency in LepRb-expressing neurons, we examined body weight in animals placed on normal chow or high-fat diet (HFD) at weaning. Both LepRb-PTP1B$^{-/-}$ and LepRb-PTP1B$^{+/-}$ mice show significantly decreased body weight on chow compared to Cre only wild type controls (Fig. 2.2A). No body weight differences were found between PTP1B fl/fl "floxed" and LepRb-Cre only controls (data not shown). In contrast to LepRb-PTP1B$^{-/-}$ mice, global PTP1B$^{-/-}$ mice are similar in body weight compared to their wild type littermate controls on chow diet as previously shown (Zabolotny et al., 2002) (Fig. 2.2B). When compared to whole-body PTP1B$^{-/-}$ mice, LepRb-specific PTP1B$^{-/-}$ mice weigh less on chow (Fig. 2.2C). On HFD, LepRb-PTP1B$^{-/-}$ mice display a significant reduction in body weight compared to wild type LepRb-Cre controls (Fig. 2.2D). Consistent with a previous report for PTP1B$^{+/-}$ mice on HFD (13), LepRb-PTP1B$^{+/-}$ mice on HFD do not display decreased body weight compared to wild type controls. Global PTP1B$^{-/-}$ mice show reduced body weight on HFD to a comparable extent as that of LepRb-PTP1B$^{-/-}$ mice, demonstrating for the first time that the protection against diet-induced obesity seen in PTP1B-deficient models is largely (if not exclusively) due to PTP1B deficiency within LepRb-expressing neurons (Fig. 2.2E, 2.2F).

LepRb-PTP1B$^{-/-}$ mice have decreased adiposity and body length on HFD.

In order to determine whether the decreased body weight of LepRb-PTP1B$^{-/-}$ mice reflects decreases in fat mass or linear growth, we measured epididymal fat pad weights and body length. LepRb-PTP1B$^{-/-}$ mice on chow diet show a non-significant trend towards decreased adiposity as measured by epididymal fat pad weight when compared to Cre-only wild type mice (Cre vs. LepRb-PTP1B$^{-/-}$ 0.368 ± 0.083g vs. 0.278 ± 0.036g, p =.341) (Fig. 2.3A). In contrast, global PTP1B$^{-/-}$ mice have decreased adiposity compared to controls despite similar body weight (Fig. 2.3B, PTP1B^{+}/$^{+}$ vs. PTP1B$^{-/-}$; 0.410 ± 0.059g vs. 0.193 ± 0.020g, p< 0.01). On HFD, adiposity is significantly decreased in LepRb-PTP1B$^{-/-}$, LepRb-PTP1B$^{+/-}$, and global PTP1B$^{-/-}$ mice at 18 weeks of age compared to their respective controls (Fig. 2.3A, 2.3B).

Body length of LepRb-PTP1B$^{-/-}$ mice on chow is unchanged when compared to Cre-only wild type controls (Fig. 2.3C). Likewise, whole-body PTP1B$^{-/-}$ mice show no difference in body length compared to their respective controls on chow (Fig. 2.3D). On HFD, LepRb-PTP1B$^{-/-}$ and LepRb-PTP1B$^{+/-}$ mice display significantly decreased body length compared to Cre-only controls (Fig. 2.3C). Similarly, whole-body PTP1B$^{-/-}$ on HFD also show decreased linear growth compared to controls (Fig. 2.3D).

Food intake and core temperature in LepRb-PTP1B$^{-/-}$ mice.

In order to determine the cause of the reduced body weight and adiposity in LepRb-PTP1B$^{-/-}$ mice, we measured daily food intake. Average daily food intake on both chow (Fig. 2.4A) and HFD (data not shown) is slightly decreased in LepRb-PTP1B$^{-/-}$ compared to Cre-only wild types although these differences do not reach statistical significance. To more carefully assess food intake over time, cumulative chow food intake over the course of 1 week was measured. Cumulative food intake is decreased in LepRb-PTP1B$^{-/-}$ mice compared to controls on chow (Fig. 2.4B). In contrast, global PTP1B$^{-/-}$ mice have increased average daily and cumulative food intake on chow, consistent with past reports (Klaman et al., 2000) (Fig. 2.4C and 2.4D).

Global, brain-specific, and POMC neuron-specific PTP1B$^{-/-}$ models exhibit a lean metabolic phenotype and increased energy expenditure (Elchebly et al., 1999; Klaman et al., 2000; Bence et al., 2006; Banno et al., 2010). To assess whether LepRb-PTP1B$^{-/-}$ mice showed enhanced diet-induced adaptive thermogenesis, we measured core temperature after placing adult animals (8 weeks of age) on a HFD for 2 weeks. Core temperature of LepRb-PTP1B$^{-/-}$ mice is elevated by more than 0.5°C although this does not reach statistical significance (Fig. 2.4E, Cre vs. LepRb-PTP1B$^{-/-}$; 36.83 ± 0.39°C vs. 37.43 ± 0.16°C, p = .183). Since brown adipose tissue (BAT) oxidizes chemical energy and is the central tissue promoting thermogenesis, we examined expression of uncoupling protein 1 (UCP1) in BAT. Interscapular BAT UCP1 expression is unchanged in LepRb-PTP1B$^{-/-}$ mice compared to Cre-only controls (data not shown). Although no differences were seen in BAT UCP1 expression, we measured expression of BAT gene markers in epididymal white adipose to see if LepRb-PTP1B$^{-/-}$ mice showed increased "browning" of white fat, which would indicate enhanced energy expenditure (Seale et al., 2007, 2011; Ishibashi and Seale, 2010; Petrovic et al., 2010). Interestingly, in chow fed animals the brown fat markers, Cidea and UCP1, show a trend towards increased expression in the epididymal white fat of LepRb-PTP1B$^{-/-}$ mice compared to Cre-only controls (Fig. 2.4F, All values Cre vs. LepRb-PTP1B-/-; Cidea 1.098 ± 0.170 vs. 1.711 ± 0.339, p = .162; UCP1 1.108 ± 0.167 vs. 3.227 ± 1.335, p = .159).

LepRb-PTP1B$^{-/-}$ mice show enhanced leptin sensitivity.

Mice with whole-body, brain-specific, or POMC neuron-specific PTP1B-deficiency demonstrate leptin hypersensitivity (Cheng et al., 2002; Zabolotny et al., 2002; Bence et al., 2006; Banno et al., 2010). Given the leptin receptor expressing cell-specific deletion of PTP1B in this model, we expected LepRb-PTP1B$^{-/-}$ mice to recapitulate this improvement in leptin sensitivity. No changes in food intake or body weight were noted in any genotype in response to saline injection (Fig. 2.5A and 2.5B). In response to leptin treatment (0.5μg/g/injection, i.p.), food intake is suppressed

to a similar extent in LepRb-PTP1B$^{-/-}$, PTP1B$^{-/-}$, and wild type controls (Fig. 2.5A and 2.5C). However, LepRb-PTP1B$^{-/-}$ and PTP1B$^{-/-}$ mice show a significantly greater suppression of body weight compared to their respective wild type controls (Fig. 2.5B and 2.5D), consistent with our hypothesis that LepRb-PTP1B$^{-/-}$ mice display a similar level of leptin hypersensitivity as whole-body PTP1B$^{-/-}$ mice. Additionally, we measured serum leptin levels from overnight fasted mice on chow or HFD. On chow diet, serum leptin levels are unchanged in LepRb-PTP1B$^{-/-}$ mice compared to Cre-only wild types, while global PTP1B$^{-/-}$ mice show decreased serum leptin compared to PTP1B wild types, reflecting their reduced adiposity on chow (Fig. 2.5C). On HFD, serum leptin levels are reduced in LepRb-PTP1B$^{-/-}$ mice compared to Cre-only wild types and in global-PTP1B$^{-/-}$ compared to their respective controls (Fig. 2.5D Cre vs. LepRb-PTP1B$^{-/-}$; 51.55 ± 4.33ng/ml vs 24.87 ± 4.96ng/ml, $p < 0.01$, PTP1B$^{+/+}$ vs PTP1B$^{-/-}$; 27.36 ± 3.56 vs 7.602 ± 1.71, $p < 0.01$).

LepRb-PTP1B$^{-/-}$ mice show improvements in glucose homeostasis.

Since we previously found that neuronal deficiency of PTP1B can improve peripheral glucose homeostasis (Bence et al., 2006; Banno et al., 2010), we examined glucose tolerance in LepRb-PTP1B$^{-/-}$ mice compared to controls. On chow, LepRb-PTP1B$^{-/-}$ mice display similar glucose clearance compared to Cre only controls as measured by a glucose tolerance test (GTT), and have similar fasting serum insulin levels compared to controls (Fig. 2.6A and 2.6C). On HFD, however, LepRb-PTP1B$^{-/-}$ show significantly improved glucose tolerance and fasting serum insulin compared to Cre only controls (Fig. 2.6B and 2.6D, Cre vs. LepRb-PTP1B$^{-/-}$; 2.076 ± 0.264ng/ml vs. 0.826 ± 0.087ng/ml, $p < 0.01$). As expected, global PTP1B$^{-/-}$ mice also show significantly enhanced glucose clearance and lower fasting serum insulin levels compared to controls (PTP1B$^{+/+}$ vs. PTP1B$^{-/-}$; 1.987 ± 0.535ng/ml vs. 0.792 ± 0.135ng/ml, $p < 0.01$), and displayed an additional enhancement in glucose clearance compared to LepRb-PTP1B$^{-/-}$ mice most likely due to the additional deletion of PTP1B in muscle and liver (Elchebly et al., 1999; Klaman et al., 2000; Delibegovic et al., 2007, 2009).

Discussion

The extent to which PTP1B's metabolic effects are mediated exclusively via LepRb-expressing neurons was previously unknown. Our data clearly demonstrate an important role for PTP1B in LepRb-expressing neurons in the control of body weight and leptin sensitivity in mice. Additionally, our findings highlight subtle differences between PTP1B's metabolic role in the whole body compared to its restricted effects within leptin receptor expressing neurons, suggesting an additional leptin-independent metabolic role for PTP1B.

Global PTP1B-deficiency, as well as neuronal PTP1B-deficiency, results in a lean metabolic phenotype with decreased HFD-induced adiposity in mice (Klaman et al., 2000; Bence et al., 2006). We demonstrate that LepRb-PTP1B$^{-/-}$ mice display a significant decrease in body weight and adiposity when fed a HFD, which overall is indistinguishable from global PTP1B$^{-/-}$ mice. Thus, the lean metabolic phenotype of global PTP1B$^{-/-}$ mice fed a HFD likely results from PTP1B deficiency within LepRb-expressing neurons. Similar to mice with neuronal PTP1B-deficiency, LepRb-PTP1B$^{-/-}$ mice fed a chow diet weighed less than wild type controls and global PTP1B$^{-/-}$ mice. However, global PTP1B$^{-/-}$ mice do not show decreased body weight on chow diet. Previous studies have found that muscle-, liver-, or adipocyte-specific deletion of PTP1B does not result in decreased body weight or adiposity, while deletion of PTP1B in adipocytes and macrophages using the aP2-driven cre results in weight gain (Bence et al., 2006; Delibegovic et al., 2007, 2009; Owen et al., 2012). The lack of any body weight difference in chow-fed global PTP1B$^{-/-}$ mice compared to controls may result from the combined effects of PTP1B deficiency in both brain and macrophages; this interesting possibility remains to be explored. Interestingly, global PTP1B$^{-/-}$ mice have reduced adiposity on chow diet despite similar body weight as wild types, whereas LepRb-PTP1B$^{-/-}$ mice show a trend toward reduced adiposity on chow, despite lower body weight.

The decreased body weight and adiposity of LepRb-PTP1B$^{-/-}$ mice are potentially a combined result of decreased food intake over time and increased energy expenditure due to enhanced leptin sensitivity. Indeed, cumulative food intake is lower in LepRb-PTP1B$^{-/-}$ mice, and core temperature and markers of white adipose "browning" tend to be higher. Overall these differences are subtle in magnitude, however this phenotype is consistent within the context of other PTP1B-deficient models which show enhanced energy expenditure measured by indirect calorimetry (Klaman et al., 2000; Bence et al., 2006; Banno et al., 2010), and is also consistent with the fact that the reduction in body weight occurs slowly over time.

Previous models of PTP1B-deficiency have all demonstrated clear elevations in energy expenditure (Klaman et al., 2000; Bence et al., 2006; Banno et al., 2010) whereas only brain-specific PTP1B$^{-/-}$ mice showed a decrease in food intake. Other neuronal populations which express PTP1B (separate from LepRb- expressing-neurons) may also play a role in food intake regulation, explaining the more pronounced suppression in food intake seen in complete brain-specific PTP1B knockouts. For example, insulin has been shown to have food intake reducing effects (Woods et al., 1979; Schwartz et al., 1992; McGowan et al., 1993; Air et al., 2002b; Carvalheira et al., 2003), and may act upon cell populations both overlapping and distinct from LepRb-expressing populations. The insulin receptor (IR) is broadly expressed within the CNS including the hypothalamus. Moreover, PTP1B is a known regulator of insulin signaling via dephosphorylation of IR and IRS (Seely et al., 1996; Bandyopadhyay et al., 1997; Byon et al., 1998; Goldstein et al., 2000; Dadke and Chernoff, 2002). Although PTP1B deletion in LepRb neurons may in turn sensitize insulin signaling pathways, the extent to which LepRb and IR are coexpressed within the CNS is unclear, and in the case of POMC neurons, leptin and insulin appear to signal in separate, distinct subpopulations (Williams et al., 2010).

Like global-, brain-, and POMC-PTP1B$^{-/-}$ mice, LepRb-PTP1B$^{-/-}$ also display improved glucose tolerance and decreased circulating insulin levels on HFD. Whether these indications of

improved insulin sensitivity are independent of differences in body weight and adiposity is unclear given LepRb-PTP1B$^{-/-}$ mice exhibit decreased body weight on chow and HFD. There is precedent for central PTP1B regulation of peripheral insulin sensitivity; POMC-PTP1B$^{-/-}$ mice display improved insulin sensitivity even when controlled for body weight and adiposity. Given the degree of overlap (at the neuronal level) in expression of POMC and LepRb, regulation of peripheral insulin sensitivity by neuronal PTP1B may, at least partially, account for the improvements seen in LepRb-PTP1B$^{-/-}$ mice. Central leptin signaling has been closely tied to the control of peripheral glucose homeostasis (Morton, 2007; Li et al., 2011), and enhancing central leptin sensitivity can improve insulin sensitivity at the periphery. Like PTP1B deficiency, deletion of suppressor of cytokine signaling-3, a separate known negative regulator of leptin signaling, results in improved peripheral glucose homeostasis (Mori et al., 2004; Kievit et al., 2006). Thus, enhanced leptin sensitivity of LepRb-PTP1B$^{-/-}$ mice may underlie the improvements seen in glucose tolerance and fasting insulin levels.

The subtle metabolic differences between LepRb-PTP1B$^{-/-}$ and global PTP1B$^{-/-}$ mice point towards a distinct, metabolically significant role for PTP1B outside of leptin receptor expressing neurons. The significant reduction in adiposity seen in chow-fed global PTP1B$^{-/-}$ compared to LepRb-PTP1B$^{-/-}$ mice may be explained by PTP1B deficiency in non-LepRb expressing neurons and/or in other tissues of global PTP1B$^{-/-}$ mice. For example, PTP1B has been shown to regulate BAT adipogenesis; preadipocyte cell lines derived from PTP1B$^{-/-}$ mice show enhanced BAT differentiation when compared to wild type-derived cell lines (Miranda et al., 2010; Matsuo et al., 2011). PTP1B deletion in whole body or brain results in increased activity of the fuel sensing enzyme AMPK as well as increased UCP1 expression in BAT; increased AMPK activity in BAT promotes expression of fatty acid oxidation and mitochondrial markers (Xue et al., 2009). Additionally, non-leptin signaling pathways that play a role in the central control of energy homeostasis may be sensitized in non-LepRb expressing neurons, potentially contributing to the reduced adiposity phenotype limited to global PTP1B$^{-/-}$ mice on chow. It should be noted that

although both LepRb-PTP1B$^{-/-}$ and global PTP1B$^{-/-}$ mice are on a mixed 129Sv/J × C57BL/6 background, the two lines were generated in parallel, and subtle differences in genetic strain may also contribute to the metabolic differences observed.

In models of diet-induced obesity, whether leptin resistance is a cause or consequence of the obese state is incompletely understood. Current evidence support leptin resistance as a consequence and subsequent facilitator of the obese state: HFD fed mice exhibit baseline increases in arcuate pSTAT3 levels in absence of exogenous leptin (Björnholm et al., 2007). Diet-induced obese animals tend to reduce food intake and body weight after removal of the palatable diet, thus cellular leptin resistance is not enough to maintain the obese phenotype. In a model of obesity proposed by Myers et al., increased caloric intake promotes weight gain, increased adiposity, and cellular leptin resistance; the development of leptin resistance in turn facilitates further weight gain (Myers et al., 2010). This model suggests that cellular leptin resistance develops over time, therefore the benefits of increased leptin sensitivity from LepRb-specific PTP1B deficiency would not be immediately noticeable in young animals. In spite of this, global PTP1B$^{-/-}$ mice on HFD show body weight differences early on, near weaning age (Klaman et al., 2000). Although these differences are small, our data was consistent with Klaman et al. 2000, with global PTP1B$^{-/-}$ mice displaying significant differences in body weight appearing as early as 1-2 weeks post weaning (Fig. 2.2E). On the other hand, LepRb-PTP1B$^{-/-}$ mice display decreased body weight on HFD at 7 weeks post weaning (10 weeks of age). This early weight difference between global PTP1B$^{-/-}$ and LepRb-PTP1B$^{-/-}$ mice may be explained by PTP1B outside of LepRb-expressing neurons sensitizing non-leptin signaling pathways which may affect early growth and development. Alternatively, the temporal expression pattern of PTP1B within LepRb-expressing neurons may be affecting early growth. For example, LepRb-PTP1B$^{-/-}$ mice are capable of expressing PTP1B in LepRb-expressing progenitors *until* LepRb expression occurs during development. LepRb expression in the brain occurs as early as embryonic day 14 (Matsuda et al., 1999), however global PTP1B$^{-/-}$ mice lack PTP1B in all neurons throughout

development. Thus, the early loss of PTP1B in LepRb progenitors and/or non-LepRb neurons may account for the small, early weight differences seen between global PTP1B$^{-/-}$ and LepRb-PTP1B$^{-/-}$ mice.

Taken together, the data here suggest that PTP1B has both leptin-dependent and -independent metabolic roles. Deletion of PTP1B in LepRb-expressing neurons clearly results in increased leptin sensitivity and a lean metabolic phenotype, consistent with previous models of targeted PTP1B-deficiency *in vivo*. While PTP1B deficiency in LepRb- expressing neurons encompasses a majority of the metabolic phenotype in PTP1B-deficient models, differences between LepRb-PTP1B$^{-/-}$ and global PTP1B$^{-/-}$ mice suggest PTP1B's contribution to energy homeostasis regulation also extends outside of leptin signaling.

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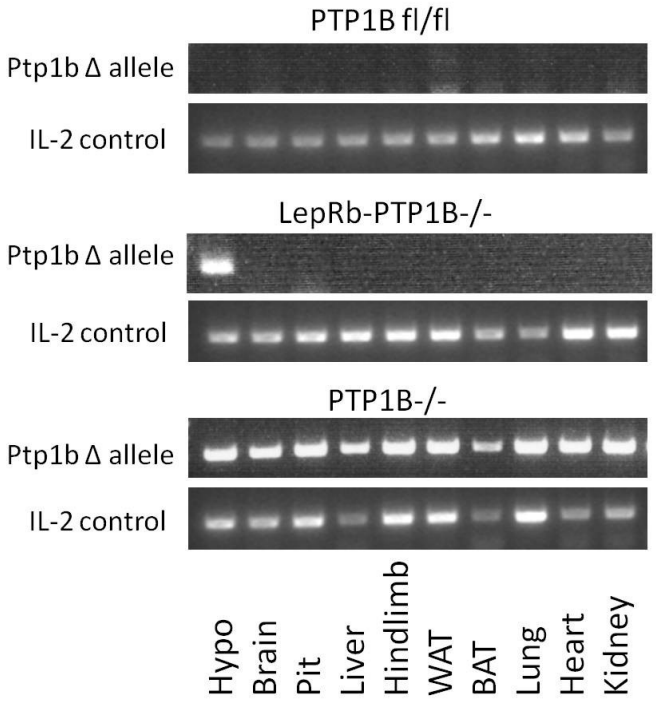

Figure 2.1 Detection of deletion of PTP1B floxed allele in LepRb-PTP1B-/- mice. DNA was isolated from different tissues [hypothalamus (Hypo), brain without hypothalamus, pituitary (Pit), liver, hindlimb, white adipose tissue (WAT), BAT, lung, heart, and kidney], and deletion of floxed allele was detected by PCR.

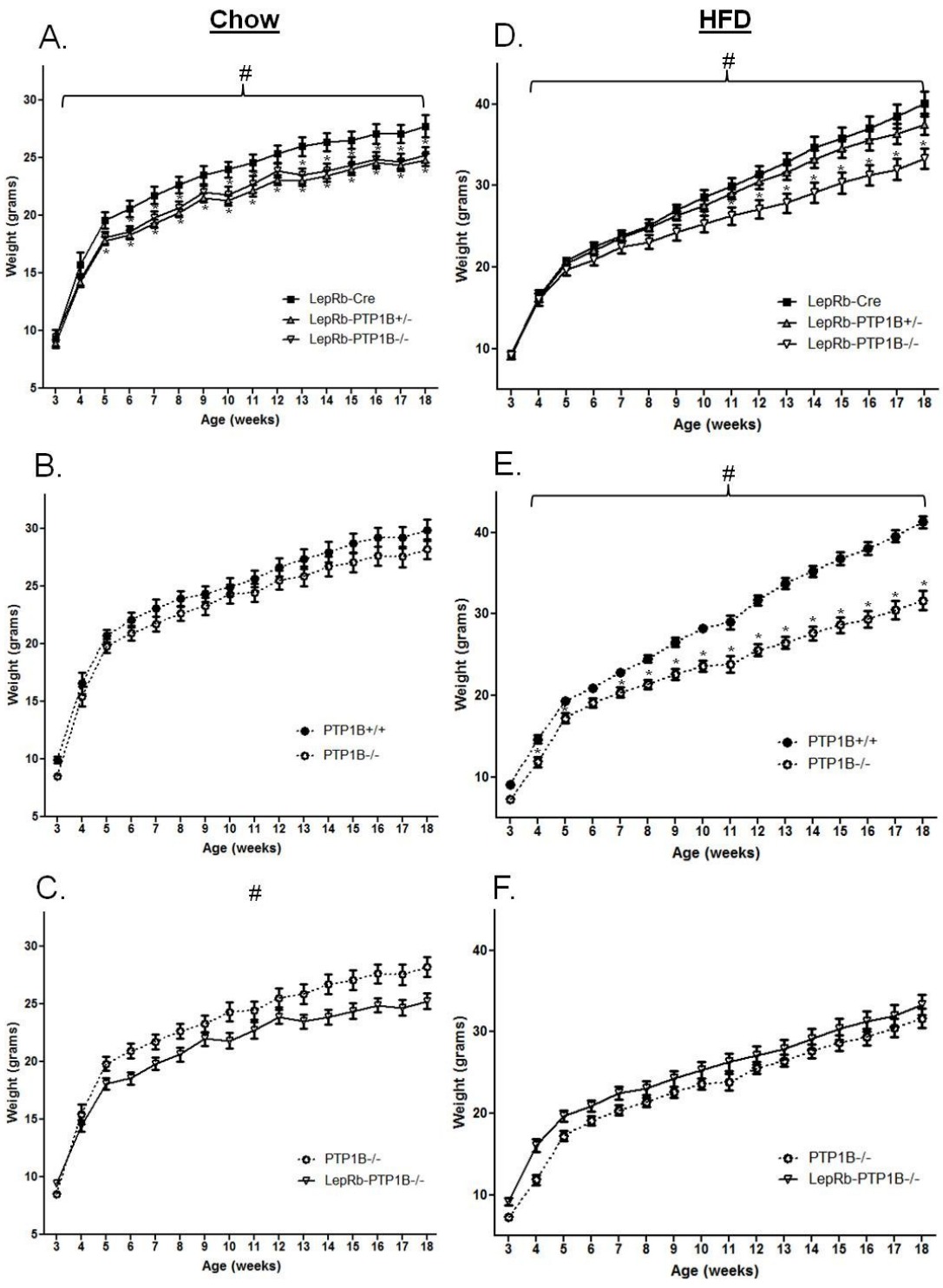

Figure 2.2 LepRb-PTP1B-/- mice have reduced body weight on chow and HFD.

Figure 2.2 cont. (A) Body weights of male LepRb-PTP1B$^{-/-}$ (n=11), LepRb-PTP1B+/- (n=16), and LepRb-Cre mice (n=9) on chow. (B) Body weights of male whole body PTP1B$^{-/-}$ (n=14) and wild type controls (n=9) on chow. (C) Body weights of male LepRb-PTP1B$^{-/-}$ and whole body PTP1B$^{-/-}$ mice on chow. (D) Body weights of male LepRb-PTP1B$^{-/-}$ (n=13), LepRb-PTP1B$^{+/-}$ (n=23), and LepRb-Cre mice (n=16) on HFD. (E) Body weights of male whole body PTP1B$^{-/-}$ (n=14) and wild type controls (n=13) on HFD. (F) Body weights of male LepRb-PTP1B$^{-/-}$ and whole body PTP1B$^{-/-}$ mice on HFD. All values are mean±SEM. Weight curves analyzed by 2-way ANOVA with repeated measures followed by pairwise comparisons: #p<0.05 by 2-way ANOVA with repeated measures. *p<0.05 by Fisher's LSD posthoc test for indicated group compared to Cre (panels A, D) or wild type control (panel E). *p<0.05 by Fisher's LSD posthoc test for LepRb-PTP1B$^{-/-}$ compared to PTP1B$^{-/-}$ mice (panel C).

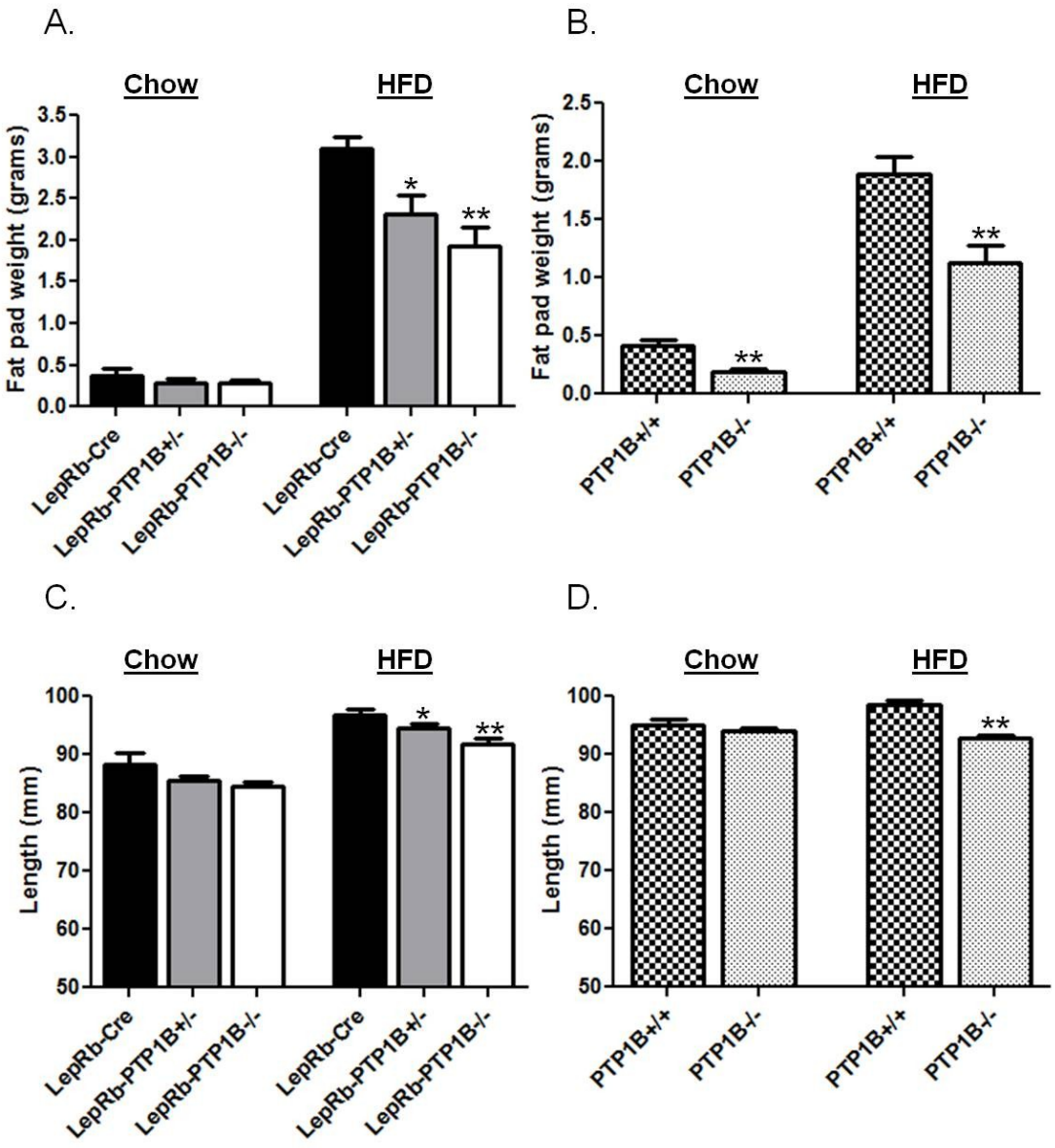

Figure 2.3 LepRb-PTP1B$^{-/-}$ mice have decreased adiposity and body length on HFD. (A) Epididymal fat pad weight of male LepRb-PTP1B$^{-/-}$ (n=10 chow, n=10 HFD), LepRb-PTP1B+/- (n=16 chow, n=9 HFD), and LepRb-Cre mice (n=9 chow, n=8 HFD). (B) Epididymal fat pad weight of male whole body PTP1B$^{-/-}$ mice (n=14 chow, n=14 HFD) and wild type controls (n=9 chow, n=7 HFD). (C) Nose-rump length of male LepRb-PTP1B$^{-/-}$ (n=6 chow, n=10 HFD), LepRb-PTP1B+/- (n=12 chow, n=9 HFD), and LepRb-Cre mice (n=6 chow, n=8 HFD). (D) Nose-rump length of male whole body PTP1B-/- mice (n=8 chow, n=14 HFD) and wild type controls (n=13 chow, n=7 HFD). All values are mean±SEM. *p<0.05 for indicated group compared to Cre or wild type control. **p<0.01 for indicated group compared to Cre or wild type control.

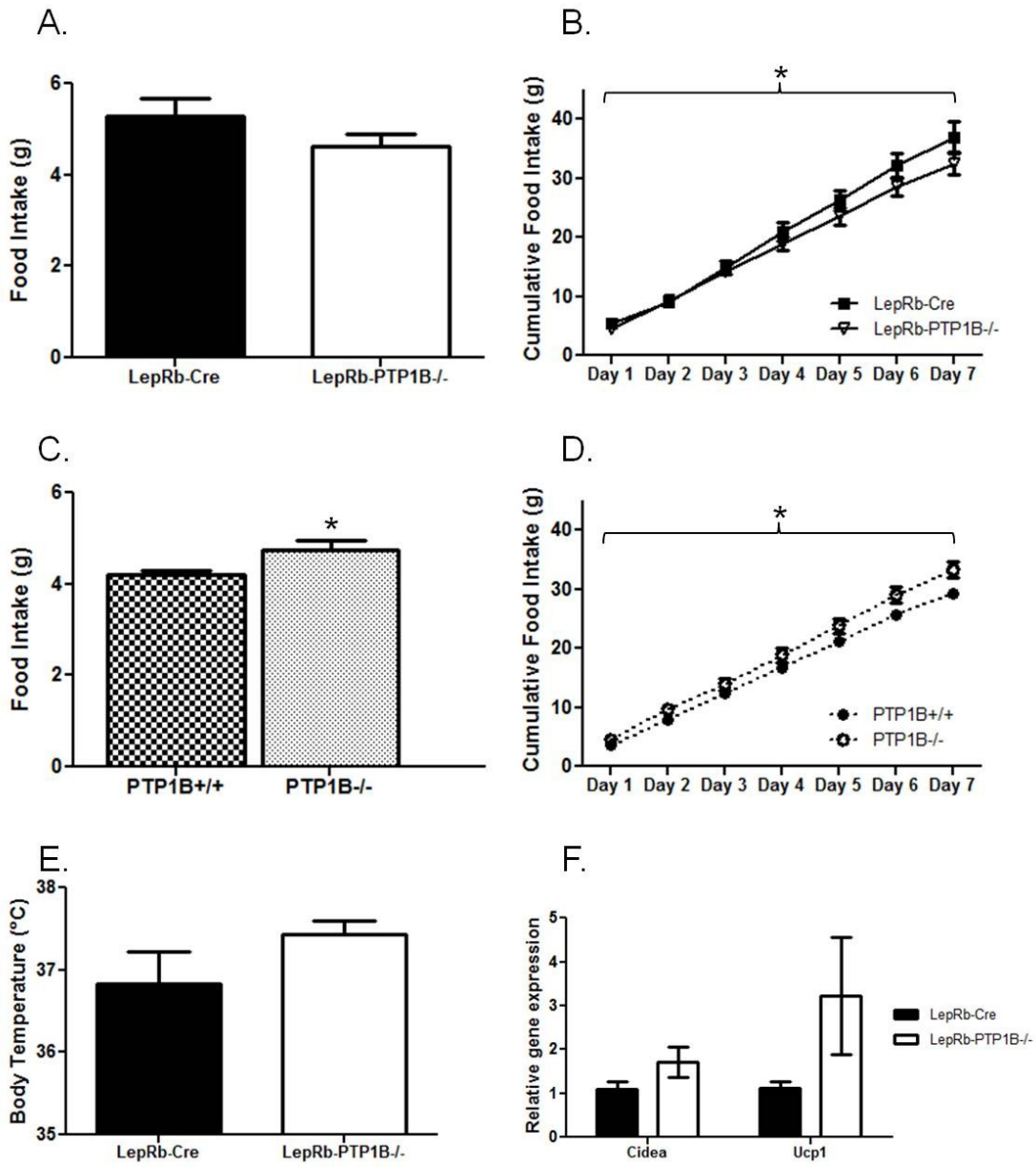

Figure 2.4 Food intake, core temperature, and expression of brown adipose tissue genes in epididymal white adipose. (A) Average daily food intake of male LepRb-PTP1B$^{-/-}$ and LepRb-Cre mice on chow (n=6/group). (B) Cumulative food intake of male LepRb-PTP1B$^{-/-}$ and LepRb-Cre mice on chow. (C) Average daily food intake of male whole body PTP1B$^{-/-}$ and wild type controls on chow (n=6/group). (D) Cumulative food intake of male whole body PTP1B$^{-/-}$ and wild type controls on chow. (E) Core temperature of male LepRb-PTP1B$^{-/-}$ and LepRb-Cre mice after 2 weeks of HFD (n=6/group). (F) Expression of *Cidea* and *Ucp1* in epididymal white adipose of male LepRb-PTP1B$^{-/-}$ and LepRb-Cre mice on chow. All values are mean±SEM. *p<0.05 for indicated group compared to Cre or wild type control. For cumulative food intake: *p<0.05 by 2-way ANOVA with repeated measures.

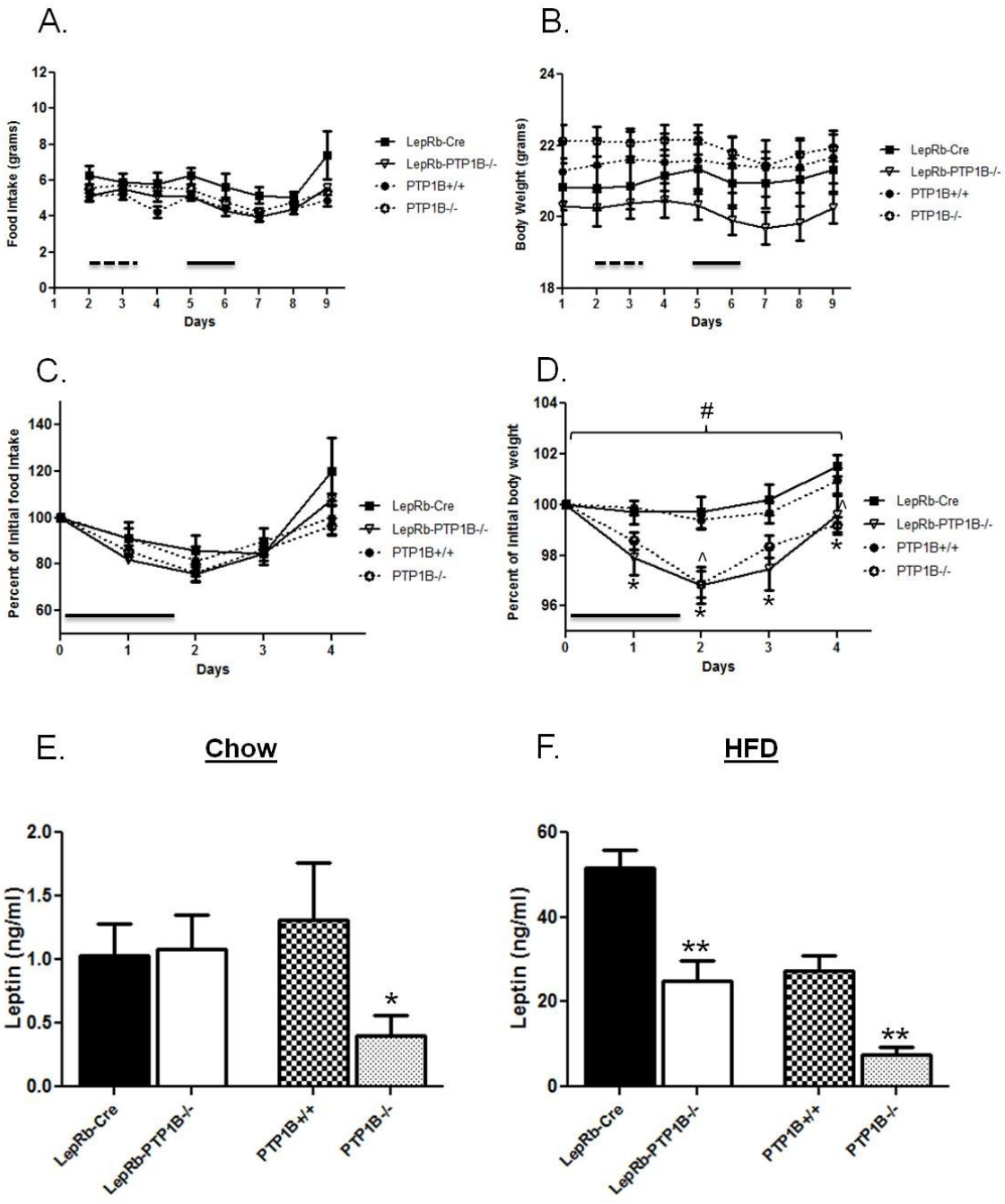

Figure 2.5 LepRb-PTP1B$^{-/-}$ mice have increased leptin sensitivity. (A) Male mice (7 weeks of age on a chow diet) were initially injected with saline i.p. every 12 hours over the course of 36 hours indicated by the dashed bar. After 24 hours of recovery, mice were injected with leptin (0.5 µg/g body weight every 12 hours for 48 hours) i.p. indicated by the solid bar (n = 6/genotype). LepRb-PTP1B$^{-/-}$, whole body PTP1B$^{-/-}$ mice, Cre and wild type controls show suppression of food intake in response to leptin.

Figure 2.5 cont. (B) Male LepRb-PTP1B$^{-/-}$ and whole body PTP1B$^{-/-}$ mice show suppression of body weight while Cre and wild type controls do not respond to low dose leptin. (C) Percent change in food intake in response to leptin injection compared to saline baseline. (D) Percent change in body weight in response to leptin injection compared to saline baseline. (E) Fasting serum leptin levels in male LepRb-PTP1B$^{-/-}$ (n=17) and LepRb-Cre mice (n=16), and whole body PTP1B$^{-/-}$ mice (n=14) and wild type controls (n=9) on chow (18 weeks old). (F) Fasting serum leptin levels in male LepRb-PTP1B$^{-/-}$ (n=7) and LepRb-Cre mice (n=8), and whole body PTP1B$^{-/-}$ mice (n=13) and wild type controls (n=7) on HFD (18 weeks old). All values are mean±SEM. Leptin sensitivity analyzed by 2-way ANOVA with repeated measures followed by pairwise comparisons: #p<0.05 by 2-way ANOVA with repeated measures. *p<0.05 by Fisher's LSD posthoc test for LepRb-PTP1B$^{-/-}$ compared to Cre control. ^p<0.05 by Fisher's LSD posthoc test for PTP1B$^{-/-}$ vs. wild type control. For serum leptin comparisons: *p<0.05 for indicated group compared to Cre or wild type control. **p<0.01 for indicated group compared to Cre or wild type control.

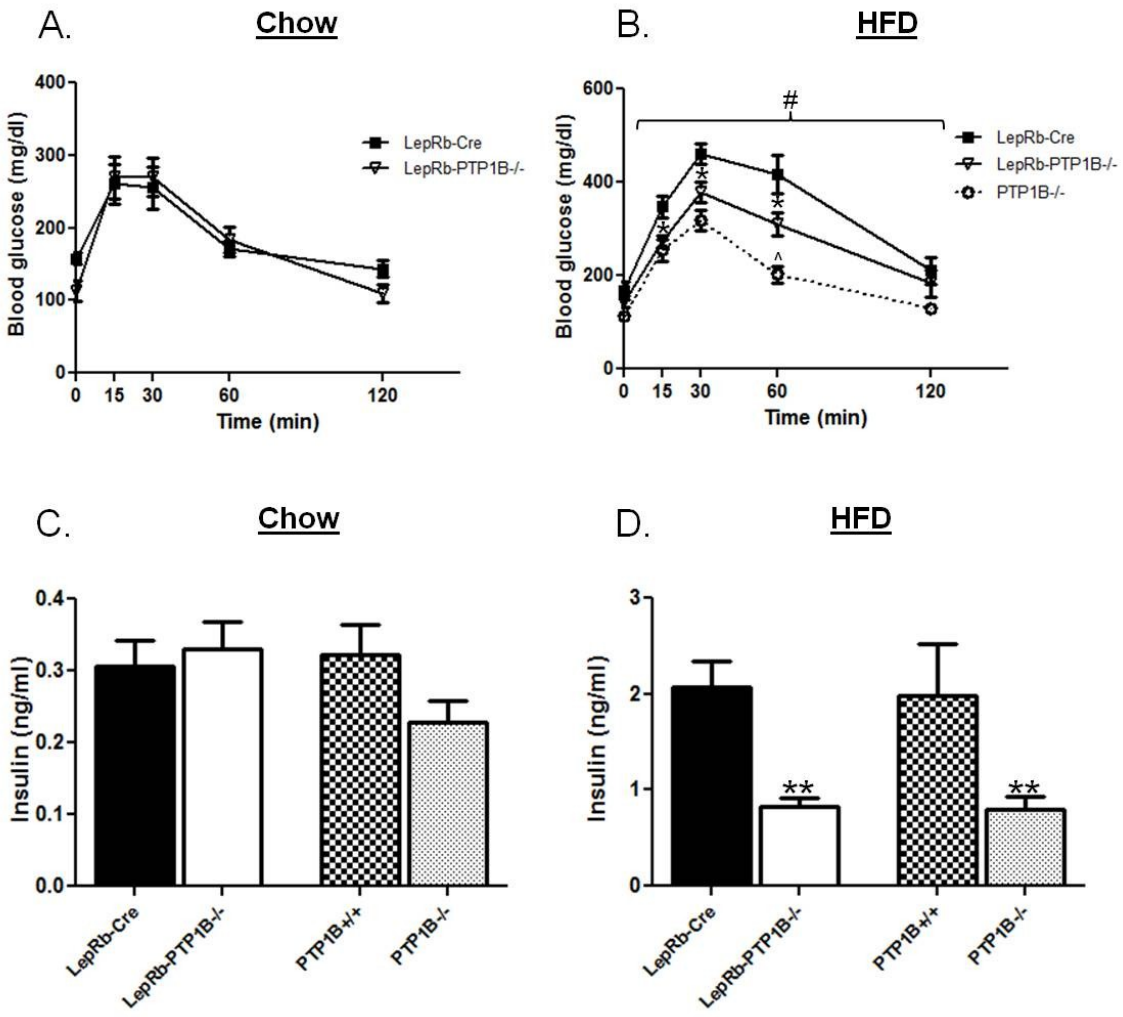

Figure 2.6 LepRb-PTP1B$^{-/-}$ mice on HFD have improved glucose tolerance. (A) GTT for male LepRb-PTP1B$^{-/-}$ (n=10), and LepRb-Cre mice (n=7) on chow (8-10 weeks old). (B) GTT for male LepRb-PTP1B$^{-/-}$ (n=8), LepRb-Cre (n=8), and whole body PTP1B$^{-/-}$ mice (n=6) on HFD (8-10 weeks old). (C) Fasting serum insulin levels in male LepRb-PTP1B$^{-/-}$ (n=17) and LepRb-Cre mice (n=16), and whole body PTP1B$^{-/-}$ mice (n=14) and wild type controls (n=9) on chow (18 weeks old). (D) Fasting serum insulin levels in male LepRb-PTP1B$^{-/-}$ (n=10) and LepRb-Cre mice (n=8), and whole body PTP1B$^{-/-}$ mice (n=14) and wild type controls (n=7) on HFD (18 weeks old). All values are mean±SEM. GTT analyzed by 2-way ANOVA with repeated measures followed by pairwise comparisons: #p<0.05 by 2-way ANOVA with repeated measures. *p<0.05 by Fisher's LSD posthoc test for LepRb-PTP1B$^{-/-}$ compared to Cre control. ^p<0.05 by Fisher's LSD posthoc test for PTP1B$^{-/-}$ vs. LepRb-PTP1B$^{-/-}$ mice. For serum insulin comparisons: **p<0.01 for indicated group compared to Cre or wild type control.

CHAPTER 3: Improved Metabolic Phenotype of Hypothalamic PTP1B-Deficiency is Dependent upon the Leptin Receptor

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Abstract

Protein tyrosine phosphatase 1B (PTP1B) is a known regulator of central metabolic signaling, and mice with whole brain-, leptin receptor (LepRb) expressing cell-, or proopiomelanocortin neuron-specific PTP1B-deficiency are lean, leptin hypersensitive, and display improved glucose homeostasis. However, whether the metabolic effects of central PTP1B-deficiency are due to action within the hypothalamus remains unclear. Moreover, whether or not these effects are exclusively due to enhanced leptin signaling is unknown. Here we report that mice with hypothalamic PTP1B-deficiency (Nkx2.1-PTP1B$^{-/-}$) display decreased body weight and adiposity on high-fat diet with no associated improvements in glucose tolerance. Consistent with previous reports, we find that hypothalamic deletion of the LepRb in mice (Nkx2.1-LepRb$^{-/-}$) results in extreme hyperphagia and obesity. Interestingly, deletion of hypothalamic PTP1B and LepRb (Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$) does not rescue the hyperphagia or obesity of Nkx2.1-LepRb$^{-/-}$ mice, suggesting that hypothalamic PTP1B contributes to the central control of energy balance through a leptin receptor-dependent pathway.

Introduction

Obesity continues to be a major public health crisis in the United States and worldwide (Flegal et al., 2012; Popkin et al., 2012; Popkin and Slining, 2013). Given the numerous metabolic comorbidities and an overall increased all-cause mortality associated with obesity (Catenacci et al., 2009; Flegal et al., 2013), understanding the underlying biological systems that regulate body weight and adiposity is of great importance. Though obesity is commonly thought of as a disease affecting the periphery (i.e. increased body fat), the central nervous system (CNS) plays a key role in regulating appetite, metabolism, and body weight. Neurons within the hypothalamus integrate neuroendocrine signals from the periphery, gauging short term and long term energy status. The adipocyte-secreted hormone leptin is one such signal whose effects on the central control of energy homeostasis have been studied in depth. Circulating leptin acts on leptin receptors (LepRbs) expressed within the hypothalamus and extrahypothalamic sites (hindbrain nucleus tractus solitarius, parabrachial nucleus) (Scott et al., 2009; Patterson et al., 2011) to suppress food intake and increase energy expenditure, ultimately promoting negative energy balance. Leptin- (*ob/ob*) and LepRb-deficient (*db/db*) mice are hyperphagic and develop extreme obesity (Zhang et al., 1994; Chua et al., 1996; Lee et al., 1996). Moreover, deletion of LepRb within the hypothalamus in mice, driven by the ventral forebrain specific Nkx2.1-Cre, recapitulates much of the *db/db* phenotype (Ring and Zeltser, 2010). LepRb-deficiency within the hindbrain NTS in mice also results in hyperphagia and increased weight gain (Scott et al., 2011).

At the molecular level, when LepRb is activated, several tyrosine phosphorylation events occur. Initially, leptin binding to LepRb results in a conformational change of the receptor and activation of the associated tyrosine kinase Janus kinase 2 (JAK2). JAK2 autophosphorylates and subsequently phosphorylates tyrosine residues along the intracellular tail of LepRb, which can further recruit downstream signaling molecules necessary for eliciting leptin's physiological effects (Bjørbaek and Kahn, 2004; Robertson et al., 2008). Protein tyrosine phosphatase 1B (PTP1B)

shows enriched expression correlating with areas of LepRb expression (Zabolotny et al., 2002), and is a known negative regulator of leptin signaling via direct dephosphorylation of JAK2 (Myers et al., 2001; Cheng et al., 2002; Zabolotny et al., 2002). Whole body, whole brain-, LepRb-expressing cell-, or POMC neuron-specific PTP1B-deficiency results in decreased body weight and adiposity on HFD (Elchebly et al., 1999; Klaman et al., 2000; Bence et al., 2006; Banno et al., 2010; Tsou et al., 2012), whereas deletion of PTP1B in peripheral tissues does not affect body weight (Delibegovic et al., 2007, 2009; Owen et al., 2012; Grant et al., 2013). Since CNS PTP1B-deficient models to date have used holistic (whole brain) or neuron specific approaches (POMC- or LepRb-targeted), the anatomic specificity of PTP1B's metabolic effects remains unclear. Like the LepRb, POMC is expressed both in the hypothalamus and hindbrain, and there is evidence of enhanced hypothalamic and hindbrain leptin signaling in POMC-PTP1B$^{-/-}$ mice (Banno et al., 2010; De Jonghe et al., 2012), suggesting a metabolic role for PTP1B in both regions. Thus, the extent to which the metabolic effects of PTP1B deficiency are due to action within the hypothalamus or in extrahypothalamic sites remains unknown. Here, to determine the metabolic contribution of hypothalamic PTP1B, we generated a genetic PTP1B deficient mouse model using the Nkx2.1-Cre line, which leads to widespread recombination within the ventral forebrain.

The improved metabolic phenotype of central PTP1B-deficient models is largely attributed to enhanced leptin sensitivity. Interestingly, however, compound *ob/ob*:PTP1B$^{-/-}$ mice show attenuated weight gain in comparison to *ob/ob* mice (Cheng et al., 2002), suggesting that there may be leptin-independent metabolic effects of PTP1B deficiency. Furthermore, *db/db*:PTP1B$^{-/-}$ mice display decreased plasma triglycerides and serum free fatty acids when compared to *db/db*:PTP1B$^{+/-}$ (Ali et al., 2009), and *ob/ob* mice treated with PTP1B antisense oligonucleotides possess decreased epididymal fat compared to saline-treated controls (Zinker et al., 2002). Thus, we were interested in examining whether or not the metabolic effects of PTP1B deficiency are exclusively leptin receptor dependent. For these studies, we crossed the Nkx2.1-Cre line with *Ptpn1*$^{loxP/loxP}$:*Lepr*$^{loxP/loxP}$ mice in order to generate compound hypothalamic Nkx2.1-PTP1B$^{-/-}$

:LepRb$^{-/-}$ mice. Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice were compared to Nkx2.1-LepRb$^{-/-}$ mice as well as wildtype controls to determine whether PTP1B's metabolic effects within the hypothalamus are dependent upon functional leptin receptor signaling.

Materials and Methods

Animal care. All animal care protocols and procedures were approved by the University of Pennsylvania Institutional Care and Use Committee. We maintained mice on a 12-hour light/12-hour dark cycle in a temperature controlled barrier facility, with free access to water and food: standard chow autoclavable Lab Diet 5010 (calories provided by protein [28.7%], fat [12.7%], and carbohydrate [58.5%]) or custom HFD Teklad TD93075 (calories provided by protein [21.2%], fat [54.8%], and carbohydrate [24%]). Age-matched littermates were used for all experiments.

Generation of Nkx2.1-PTP1B$^{-/-}$, Nkx2.1-LepRb$^{-/-}$, and Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice. All mice were on a C57BL/6 background. *Ptpn1*$^{loxP/loxP}$ mice were generated previously (Bence et al., 2006) on a mixed 129Sv/J × C57BL/6 background but were backcrossed at least 10 generations onto C57BL/6 background prior to mating with other lines. *Lepr* $^{loxP/loxP}$ mice on a C57BL/6 background were obtained from S. Chua (Albert Einstein College of Medicine) and S. Obici (University of Cincinnati, Ohio). Nkx2.1-Cre transgenic mice were obtained from The Jackson Laboratory (Stock #008661, Bar Harbor, ME). Genotyping primer sequences were as follows:

PTP1B fl forward 5′-TGCTCACTCACCCTGCTACAA

PTP1B fl reverse 5′-GAAATGGCTCACTCCTACTGG

Lepr fl forward 5′-AACGGTTTTACAGTCTCCA

Lepr fl reverse 5′-AAGGCCCATTTAGTCAAC

Nkx2.1-Cre forward 5′-CCACAGGCACCCCACAAAAATG

Nkx2.1-Cre reverse 5′-GCCTGGCGATCCCTGAACAT

Isolating DNA from tissues for detection of recombination of the floxed alleles. Tissues were digested at 55°C overnight in proteinase K digestion buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 300 µg/ml proteinase K). Saturated NaCl (~6M) was added to the digestion, and samples were vortexed vigorously for 1 minute. Samples were centrifuged for 20 minutes at 13,700xg, and supernatants were transferred to a fresh tube. DNA was precipitated by adding 1 ml 100% ethanol, and pellets were washed once with 70% ethanol and were resuspended in 100 µl of sterile PCR water for analysis. PCR primers for detection of recombined alleles: *Ptpn1*∆/∆ forward 5′-GTGGTGCCTGCAAGAGAACTGAC

Ptpn1∆/∆ reverse 5′-GAAATGGCTCACTCCTACTGG

Lepr∆/∆ forward 5′-GTCTGATTTGATAGATGGTCTT

Lepr∆/∆ reverse 5′-ACAGGCTTGAGAACATGAACAC

IL-2 internal control forward 5′-CTAGGCCACAGAATTGAAAGATCT

IL-2 internal control reverse 5′-GTAGGTGGAAATTCTAGCATCATCC

Immunoblotting. Mouse tissues were dissected and immediately frozen in liquid nitrogen. Whole cell lysates were prepared in modified RIPA buffer containing fresh protease inhibitors, and PTP1B and SHP2 immunoblotting was performed as described previously (Zabolotny et al., 2002; Banno et al., 2010). PTP1B immunoblots were normalized to SHP2 (Santa Cruz Biotechnology Inc., sc-280) to control for loading.

Body composition and food intake. At weaning, mice were placed on diets of either standard laboratory chow or HFD. Body weights were assessed weekly and food intake was measured daily at indicated age. Body length was measured as nose-rump length at indicated age. Epididymal fat pads were dissected and weighed at indicated age. Total fat and lean mass was measured in conscious mice using NMR (Echo Medical Systems) at indicated age in the Penn IDOM Mouse Phenotyping, Physiology and Metabolism Core.

Energy expenditure measurements. Rectal temperature was measured with a thermistor during the light cycle in animals at 14-17 weeks of age (MicroTherma 2T; ThermoWorks). Feed efficiency was calculated as grams weight gained/food consumed over indicated time period. Brown adipose tissue gene expression (*Ucp1* and *Prdm16*) was measured by real-time PCR as described below.

Glucose homeostasis. Glucose tolerance tests (GTTs) were performed as described previously (Klaman et al., 2000). Briefly, mice were fasted overnight (14-16 hours). Glucose dose used for intraperitoneal injections was 2 mg/g BW (20% solution). Blood glucose was assayed in tail blood using a glucometer (Contour, Bayer). Fasting insulin levels were determined as described below.

RNA extraction and real-time PCR. Mice were euthanized at the onset of the light cycle (08:00-10:00 a.m.). Tissues were rapidly dissected and flash frozen in liquid nitrogen. Total RNA was extracted from tissues using TRIzol (Invitrogen) and the RNeasy kit (QIAGEN). cDNA was synthesized from 1 µg total RNA using the Advantage RT-for-PCR kit (Clontech). The relative mRNA levels of *Ucp1* and *Prdm16* were assessed and quantified by quantitative real-time PCR (qRT-PCR). The housekeeping gene *hprt1* was used as an internal control. The qRT-PCR reactions were carried out using RT² SYBR Green qPCR Master Mix (SABiosciences), and samples were run using the Eppendorf Mastercycler ep realplex. Primer sequences for *Ucp1* and *Prdm16* were reported previously (Seale et al., 2007). Relative mRNA expression was calculated using the comparative Ct method as described previously (Bence et al., 2006).

Serum analysis. All blood samples were collected between 08:00 – 10:30am at indicated age. Fasting blood samples were collected following overnight fast (14-16 hours). Serum was separated by centrifugation at 6,000xg. Serum insulin and leptin (CrystalChem) and serum corticosterone (Immunodiagnostic Systems) were measured by ELISA.

Statistical analysis. Results are expressed as mean ± SEM. Comparisons between groups were made by unpaired 2-tailed Student's t test, 1-way ANOVA or 2-way ANOVA with repeated measures in one factor followed by Fisher's protected least significant difference (PLSD) or Student-Newman-Keuls pairwise comparison, as appropriate. A p value less than 0.05 was considered to be statistically significant.

Results

Generation of mice with hypothalamic-specific PTP1B deficiency or compound PTP1B:LepRb deficiency.

In order to generate mice with PTP1B deficiency throughout the hypothalamus, we crossed *Ptpn1*$^{loxP/loxP}$ mice to a line of transgenic Nkx2.1-Cre mice. The resulting *Ptpn1*$^{+/loxP}$:Nkx2.1-Cre mice were subsequently crossed to *Ptpn1*$^{loxP/loxP}$ mice to generate *Ptpn1*$^{loxP/loxP}$:Nkx2.1-Cre (hereafter termed Nkx2.1-PTP1B$^{-/-}$) mice, *Ptpn1*$^{+/loxP}$:Nkx2.1-Cre (hereafter termed Nkx2.1-PTP1B$^{+/-}$) mice, and *Ptpn1*$^{loxP/loxP}$ and *Ptpn1*$^{+/loxP}$ wildtype littermate controls. The Nkx2.1-Cre line has been characterized previously (Ring and Zeltser, 2010), and strong Cre recombinase expression is found broadly throughout the majority of the hypothalamus but is not detected in caudal brain sites. In addition to the hypothalamus, Nkx2.1-Cre is known to be expressed within the posterior pituitary, lung, thyroid, and the median ganglionic eminence, which may lead to limited Cre expression within migrating cortical interneurons (Xu et al., 2008). Consistent with past reports, Nkx2.1-PTP1B$^{-/-}$ mice indeed show decreased PTP1B protein expression in the hypothalamus relative to wildtype controls, but do not show differences in total PTP1B protein levels in lysates from extrahypothalamic brain (Fig. 3.1A).

To further explore whether the metabolic effects of CNS PTP1B deficiency are dependent upon functional leptin signaling, we generated mice with compound conditional deletion of PTP1B and LepRb in the hypothalamus. *Ptpn1*$^{loxP/loxP}$ mice were crossed to *Lepr*$^{loxP/loxP}$ mice to generate

Ptpn1$^{+/loxP}$:*Lepr*$^{+/loxP}$ mice. Subsequently, *Ptpn1*$^{+/loxP}$:*Lepr*$^{+/loxP}$ mice were intercrossed to generate *Ptpn1*$^{loxP/loxP}$:*Lepr*$^{loxP/loxP}$ mice. *Ptpn1*$^{loxP/loxP}$:*Lepr*$^{loxP/loxP}$ mice were then crossed with Nkx2.1-Cre mice to yield *Ptpn1*$^{+/loxP}$:*Lepr*$^{+/loxP}$:Nkx2.1-Cre mice. Finally, *Ptpn1*$^{+/loxP}$:*Lepr*$^{+/loxP}$:Nkx2.1-Cre mice were crossed to *Ptpn1*$^{loxP/loxP}$:*Lepr*$^{loxP/loxP}$ to yield *Ptpn1*$^{loxP/loxP}$:*Lepr*$^{loxP/loxP}$:Nkx2.1-Cre (hereafter termed Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$) mice and *Ptpn1*$^{loxP/loxP}$:*Lepr*$^{loxP/loxP}$ littermate, wildtype controls. In a parallel cross, Nkx2.1-LepRb$^{-/-}$ mice were generated using the same breeding strategy detailed above for Nkx2.1-PTP1B$^{-/-}$ mice. To verify deletion of the *Ptpn1* and *Lepr* genes, we extracted DNA from a variety of tissues (hypothalamus, extrahypothalamic brain, pituitary, lung, hindlimb muscle, epididymal white adipose, interscapular brown adipose, and liver) and assessed for deletion of the floxed alleles by PCR. Deletion of the *Ptpn1* and *Lepr* floxed alleles was only detected in DNA extracted from hypothalamus, extrahypothalamic brain, pituitary, and lung (Fig. 3.1B) of Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice. No deletion was detected in DNA from any tissues isolated from floxed wildtype control animals, and as expected only deletion of the *Lepr* floxed allele was detected in Nkx2.1-LepRb$^{-/-}$ mice.

Nkx2.1-PTP1B$^{-/-}$ mice have decreased body weight on high-fat diet.

We examined weekly body weights of male and female Nkx2.1-PTP1B$^{-/-}$ mice and PTP1B fl/fl controls on chow or high fat diet for at least 12 weeks from weaning. When fed a chow diet, male and female Nkx2.1-PTP1B$^{-/-}$ and Nkx2.1-PTP1B$^{+/-}$ mice have similar body weights compared to wildtype floxed controls (Fig. 3.2A and 3.2C). In contrast, HFD-fed male Nkx2.1-PTP1B$^{-/-}$ and Nkx2.1-PTP1B$^{+/-}$ mice show significantly decreased body weight compared to wildtype controls (Fig. 3.2B). Female Nkx2.1-PTP1B$^{-/-}$ and Nkx2.1-PTP1B$^{+/-}$ mice on HFD also display decreased body weight compared to controls (Fig. 3.2D), but to a lesser extent than the males. Overall, these data indicate hypothalamic PTP1B deficiency results in resistance to diet-induced obesity.

Nkx2.1-PTP1B$^{-/-}$ mice have decreased adiposity on chow or HFD.

To determine whether differences in body weight reflect decreased fat mass or total body size, body composition was assessed. Despite no difference in body weight on chow compared to wildtype controls, male Nkx2.1-PTP1B$^{-/-}$ mice display significantly decreased adiposity as determined by epididymal fat pad weight (Fig. 3.2E). Male Nkx2.1-PTP1B$^{-/-}$ mice show no difference in body length relative to controls on chow (Fig. 3.2F). Chow fed female Nkx2.1-PTP1B$^{-/-}$ mice show no difference in either perigonadal fat pad weight or body length compared to controls (Supplemental Fig. 3.1A and 3.1B). On HFD, male Nkx2.1-PTP1B$^{-/-}$ mice display decreased adiposity as measured by epididymal fat pad weight as well as total fat mass by NMR (Fig. 3.2E, 3.2G). Consistent with their reduced adiposity, male Nkx2.1-PTP1B$^{-/-}$ mice have decreased circulating leptin levels compared to wildtypes (Table 3.1). On HFD, male Nkx2.1-PTP1B$^{-/-}$ mice also show decreased lean mass and a concomitant decrease in body length (Fig. 3.2H and 3.2F). Female Nkx2.1-PTP1B$^{-/-}$ mice display decreased perigonadal fat pad weight relative to wildtypes on HFD, but show no difference in body length (Supplemental Fig. 3.1C and 3.1D).

Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice exhibit obesity on chow or HFD.

Since past CNS PTP1B$^{-/-}$ models show a lean metabolic phenotype resulting presumably from enhanced leptin sensitivity (Bence et al., 2006; Banno et al., 2010; De Jonghe et al., 2012; Tsou et al., 2012), we examined the effects of compound Nkx2.1-PTP1B:LepRb deficiency on body composition compared to Nkx2.1-LepRb$^{-/-}$ mice and wildtype controls to determine if functional leptin receptor signaling is required for any metabolic contribution of PTP1B deficiency in the hypothalamus. Male Nkx2.1-LepRb$^{-/-}$ mice and Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ both display significant weight gain compared to wildtype controls (Fig. 3.3A, chow diet and 3.3B, HFD). Interestingly, male Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ show no difference in body weight compared to Nkx2.1-LepRb$^{-/-}$ mice on chow or HFD, demonstrating that the metabolic effects of PTP1B deficiency in the hypothalamus are in fact dependent upon functional leptin receptor signaling.

Similarly, female Nkx2.1-LepRb$^{-/-}$ mice and Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ display significantly increased body weight on chow or HFD relative to controls, and the extent of weight gain is similar in Nkx2.1-LepRb$^{-/-}$ and Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice (Fig. 3.3C, chow diet and 3.3D, HFD).

Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice display increased adiposity on chow or HFD.

We examined total fat mass, lean mass, and body length in Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ and Nkx2.1-LepRb$^{-/-}$ mice on chow or HFD. On chow diet, male Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ and Nkx2.1-LepRb$^{-/-}$ mice display significantly increased total fat and lean mass compared to controls as determined by NMR (Fig. 3.4A and 3.4B). Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ and Nkx2.1-LepRb$^{-/-}$ mice also show a nonsignificant trend toward increased body length when compared to wildtype controls (Fig. 3.4C). Like body weight, the extent of increased fat mass, lean mass and length is similar between Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ and Nkx2.1-LepRb$^{-/-}$ mice on chow. Total fat mass, lean mass, and body length are also similar between Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ and Nkx2.1-LepRb$^{-/-}$ mice on HFD (Fig. 3.4D, 3.4E and 3.4F). On chow or HFD, both Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ and Nkx2.1-LepRb$^{-/-}$ mice display significantly elevated serum leptin levels consistent with their increased adiposity (Table 3.1). Similar body composition and length measures are also seen in female Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ and Nkx2.1-LepRb$^{-/-}$ mice with both groups displaying significantly increased total fat and lean mass compared to wildtype controls on chow or HFD (Supplemental Fig. 3.2 and data not shown), but no differences between the two groups.

Food intake, core temperature, and serum corticosterone measurements.

To examine the cause of the reduced body weight and adiposity observed in Nkx2.1-PTP1B$^{-/-}$ mice on HFD, we measured daily food intake and core temperature as a measure of thermogenesis. Average daily food intake and cumulative food intake on HFD is reduced in male Nkx2.1-PTP1B$^{-/-}$ compared to wildtype controls (Fig. 3.5A, 3.5B). Interestingly, feed efficiency (Δbody weight/Δfood intake) and core temperature are similar in Nkx2.1-PTP1B$^{-/-}$ and control

mice suggesting energy expenditure may not be affected in this model of hypothalamic PTP1B deficiency (Fig. 3.5C and 3.5D). Consistent with this idea, core temperature is also comparable when comparing Nkx2.1-PTP1B$^{-/-}$ and control mice at 17 weeks of age (Supplemental Fig. 3.3A). Expression of *Ucp1* in interscapular brown adipose tissue (BAT) and *Prdm16,* a gene which plays a major role in brown fat determination, in inguinal white adipose tissue (WAT) were examined to determine if BAT activation or WAT browning was enhanced in Nkx2.1-PTP1B$^{-/-}$ mice. Levels of *Ucp1* expression in BAT and *Prdm16* expression in inguinal WAT were similar in Nkx2.1-PTP1B$^{-/-}$ mice and wildtype controls (Supplemental Fig. 3.3B).

As expected from previous studies, Nkx2.1-LepRb$^{-/-}$ mice display significant hyperphagia as demonstrated by increased average daily and cumulative food intake compared to wildtype controls on chow (Fig. 3.5E and 3.5F). There were no differences in food intake across genders, thus food intake data presented is from males and females combined. Notably, Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ show similar levels of hyperphagia compared to Nkx2.1-LepRb$^{-/-}$ mice, suggesting that the food intake reduction seen in Nkx2.1-PTP1B$^{-/-}$ mice is dependent upon functional leptin receptor signaling. Feed efficiency (Fig. 3.5G, males and Supplemental Fig. 3.3C, females) is not different between Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$, Nkx2.1-LepRb$^{-/-}$, and wildtype control groups. Core temperature is similar in all three male groups (Fig. 3.5H), but female Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ and Nkx2.1-LepRb$^{-/-}$ mice show significantly decreased core temperature relative to wildtype controls (Supplemental Fig. 3.3D).

To assess whether hypothalamic PTP1B deficiency affects hypothalamus-pituitary-adrenal **(**HPA) function, we measured morning serum corticosterone levels in *ad lib* fed male mice. Nkx2.1-PTP1B$^{-/-}$ mice show similar serum corticosterone levels as wildtype controls on chow or HFD (Table 3.1). Leptin has been shown to play an important role in the regulation of the hypothalamus-pituitary-adrenal (HPA) axis, and a previous report showed significantly elevated serum corticosterone levels in Nkx2.1-LepRb$^{-/-}$ mice (Korner et al., 1999; Cohen et al., 2001;

Ring and Zeltser, 2010). Consistent with these findings, we find elevated serum corticosterone in Nkx2.1-LepRb$^{-/-}$ mice and also in Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice compared to wildtype controls on chow (Table 3.1).

Nkx2.1-PTP1B$^{-/-}$ show minor improvements in fasting HFD-induced hyperinsulinemia.

Given that past CNS-specific PTP1B deficient models show improvements in peripheral glucose tolerance and insulin sensitivity (Bence et al., 2006; Banno et al., 2010; Tsou et al., 2012), we examined glucose homeostasis in Nkx2.1-PTP1B$^{-/-}$ compared to their respective controls. Fasting blood glucose, serum insulin levels, and glucose tolerance are similar in male Nkx2.1-PTP1B$^{-/-}$ and Nkx2.1-PTP1B$^{+/-}$ mice compared to wildtype controls on chow (Table 3.1, Figs. 3.6A, 3.6B). On HFD, fasting blood glucose is comparable in male Nkx2.1-PTP1B$^{-/-}$, Nkx2.1-PTP1B$^{+/-}$ and wildtype mice, whereas fasting serum insulin is significantly decreased in Nkx2.1-PTP1B$^{+/-}$ mice and trending toward a decrease in Nkx2.1-PTP1B$^{-/-}$ mice compared to controls (Table 3.1). Despite reduced serum insulin levels on HFD, however, Nkx2.1-PTP1B$^{-/-}$ and wildtype controls perform similarly in an intraperitoneal GTT (Fig. 3.6C and 3.6D) and an ITT (data not shown). Taken together, these results show that Nkx2.1-PTP1B deficiency results in slight protection against HFD-induced hyperinsulinemia but no overt effects on overall glucose homeostasis.

Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice show severe impairments in glucose homeostasis.

Hypothalamic deficiency of LepRb results in severely impaired glucose homeostasis marked by elevated blood glucose and serum insulin levels and slower glucose clearance during a GTT (Ring and Zeltser, 2010). Although there are no differences in body weight or composition between Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ and Nkx2.1-LepRb$^{-/-}$ mice, we examined whether the additional PTP1B deficiency in Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice could have any beneficial effect on glucose homeostasis independent of energy balance when compared to Nkx2.1-LepRb$^{-/-}$ mice. As expected, obese Nkx2.1-LepRb$^{-/-}$ mice show a trend toward increased fasted blood

glucose levels on chow diet with a significant elevation in serum insulin levels (Table 3.1). Surprisingly, Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice show no protection against hyperglycemia and hyperinsulinemia, and if anything, have further impairments in these parameters compared to Nkx2.1-LepRb$^{-/-}$ mice (Table 3.1). Glucose tolerance is severely impaired in Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ and Nkx2.1-LepRb$^{-/-}$ mice on chow diet compared to wildtype controls, and the extent of glucose intolerance is similar between Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ and Nkx2.1-LepRb$^{-/-}$ mice (Fig. 3.6E and 3.6F). On HFD, Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ and Nkx2.1-LepRb$^{-/-}$ mice display similarly elevated fasted blood glucose and increased fasting serum insulin compared to wildtypes (Table 3.1). Taken together, these data demonstrate that hypothalamus-targeted PTP1B deficiency cannot rescue impaired peripheral glucose homeostasis in the context of hypothalamic leptin receptor-deficiency.

Discussion

The anatomical specificity of PTP1B's metabolic contribution within the CNS is unclear since past studies examined the effects of central PTP1B deficiency using holistic (all neurons) or cell-type specific approaches (POMC-specific, LepRb-specific). Furthermore, while central PTP1B deficiency clearly enhances leptin sensitivity, whether or not the metabolic benefits from CNS PTP1B-deficiency are solely the result of enhanced leptin signaling is unknown. Our data clearly demonstrate an important role for hypothalamic PTP1B in the central control of energy homeostasis in mice. Additionally, our findings clearly show that the metabolic improvements observed with hypothalamic PTP1B deficiency are dependent upon functional leptin receptor signaling.

Whole body, whole brain, LepRb-expressing cell or POMC-neuron specific PTP1B deficiency results in decreased body weight and adiposity on HFD (Elchebly et al., 1999; Klaman et al., 2000; Bence et al., 2006; Banno et al., 2010; Tsou et al., 2012). Consistent with past findings, we demonstrate that hypothalamic PTP1B-deficiency reduces body weight and adiposity in mice on

HFD. Somewhat surprisingly, the extent of the reduced body weight in hypothalamic PTP1B knockouts is more modest compared to the whole body, whole brain, and LepRb-expressing cell-specific PTP1B-deficient models despite the hypothalamus being long regarded as the major control center for energy balance. While neuronal and LepRb-specific PTP1B$^{-/-}$ mice show suppressed body weight gain even on a chow diet (Bence et al., 2006; Tsou et al., 2012), Nkx2.1-PTP1B$^{-/-}$ mice do not show any body weight phenotype on chow, suggesting that genetic deletion of PTP1B in the hypothalamus is not enough to confer reductions in body weight when energy balance is not being pushed towards weight gain. Likewise, deleting PTP1B solely within POMC neurons (representing a subpopulation of all LepRb-expressing neurons and a subset of all hypothalamic neurons) also results in no effect on body weight on chow diet (Banno et al., 2010). Thus, the anatomic specificity of central PTP1B's total metabolic contribution clearly includes the hypothalamus (Fig. 3.7), but also extrahypothalamic sites as the additional CNS deletion in pan-neuronal and LepRb-expressing cell specific PTP1B$^{-/-}$ models results in decreased body weights even on chow diet. Evidence of hindbrain PTP1B contribution has been demonstrated using the POMC-specific PTP1B$^{-/-}$ model, whereby hindbrain (4th ventricle) administration of leptin resulted in enhanced food intake and body weight suppression in POMC-PTP1B$^{-/-}$ mice relative to wildtype controls (De Jonghe et al., 2012). Importantly, despite no difference in body weight on chow, male Nkx2.1-PTP1B$^{-/-}$ mice show significantly decreased epididymal fat, suggesting that hypothalamic PTP1B can regulate adiposity independent of body weight. Although the decreased body weight of Nkx2.1-PTP1B$^{-/-}$ mice on HFD is primarily due to decreased fat mass, Nkx2.1-PTP1B$^{-/-}$ mice also display a small decrease in lean mass and body length. Indeed, past CNS PTP1B-deficient models have demonstrated small decreases in linear growth, and there is evidence of PTP1B's regulation of melanocortin action (Banno et al., 2010; De Jonghe et al., 2012; Tsou et al., 2012). In addition to the hypothalamus, the Nkx2.1 promoter drives Cre expression in non-hypothalamic tissues including the pituitary and thyroid. Given that these regions are implicated in neuroendocrine control of metabolism, it raises the possibility that PTP1B-deficiency in these tissues may contribute to the observed metabolic phenotypes.

However, our previous work has shown that pituitary-targeted *Ptpn1* deletion does not result in any metabolic phenotype (Banno et al., 2010), and thyroid function in whole body and POMC neuron-specific PTP1B$^{-/-}$ mice is normal (Klaman et al., 2000; De Jonghe et al., 2011a), suggesting that the metabolic effects reported here are in fact mediated through the hypothalamus.

The improved metabolic phenotype of central PTP1B-deficient models have been explained by increases in energy expenditure or a combination of suppressed food intake and increased energy expenditure (Klaman et al., 2000; Bence et al., 2006; Banno et al., 2010; Tsou et al., 2012). Like whole brain and LepRb-expressing cell specific PTP1B knockouts, Nkx2.1-PTP1B$^{-/-}$ mice demonstrate decreased food intake. Thus, hypothalamic PTP1B deficiency likely contributes to the decreased food intake seen in the neuronal and LepRb-PTP1B$^{-/-}$ mice; this is consistent with the fact that decreasing hypothalamic PTP1B via 3rd ventricle antisense oligonucleotide treatment results in significantly reduced food intake in rats (Picardi et al., 2008). Interestingly, no changes in energy expenditure are detected in Nkx2.1-PTP1B$^{-/-}$ mice as determined by feed efficiency, core temperature, or expression of BAT *Ucp1*, suggesting that the energy expenditure effects of CNS PTP1B deficiency may be localized to extrahypothalamic sites. One such site may be hindbrain LepRb/POMC-expressing neurons since both LepRb- and POMC-specific PTP1B knockouts display increased energy expenditure (Banno et al., 2010; Tsou et al., 2012). Indeed, hindbrain leptin administration in POMC-PTP1B$^{-/-}$ mice results in greater increases in spontaneous activity and core temperature compared to wildtype controls (De Jonghe et al., 2012). Alternatively, PTP1B might have competing opposing effects on energy expenditure within different subpopulations of neurons in the hypothalamus; this possibility remains to be explored.

In addition to affecting energy balance, central PTP1B has been implicated in the regulation of peripheral glucose homeostasis (Bence et al., 2006; Banno et al., 2010; Tsou et al., 2012).

Whereas whole brain, LepRb-expressing cell-, and POMC neuron-specific PTP1B$^{-/-}$ mice display significant improvements in glucose tolerance, serum insulin levels, and insulin sensitivity, hypothalamic PTP1B deficiency has no effect on glucose tolerance and only displays modest effects on fasting insulin levels. Therefore, PTP1B deficiency in extrahypothalamic LepRb and POMC neurons, likely in the hindbrain, may account for the improvements in glucose homeostasis observed in other CNS-PTP1B deficient models.

Hypothalamic LepRb deletion in mice results in significantly increased weight gain and adiposity similar to that seen in *db/db* mice (Ring and Zeltser, 2010). We demonstrate that compound PTP1B and LepRb deficiency in the hypothalamus results in weight gain comparable to that of Nkx2.1-LepRb$^{-/-}$ mice on both chow and HFD. Consistent with the results of Ring et al. 2010, we also observed that female Nkx2.1-LepRb$^{-/-}$ and Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice had increased body weight gain relative to males. In addition to body weight, body composition and length is similar between Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ and Nkx2.1-LepRb$^{-/-}$ mice. Both groups similarly demonstrate significant hyperphagia and insulin resistance as demonstrated by elevated fasting serum insulin and glucose intolerance. Interestingly, on chow, Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice display significantly elevated fasting insulin relative to Nkx2.1-LepRb$^{-/-}$ mice, suggesting that knocking out PTP1B on top of LepRb deficiency in the hypothalamus may intensify already impaired glucose homeostasis. However, whether or not a significant statistical difference translates to a relevant physiological difference is unclear as both models demonstrate impairments to glucose homeostasis several fold greater than wildtypes, and the same difference was not observed on HFD. It has been demonstrated that ventromedial hypothalamus (VMH)-specific insulin receptor deletion leads to protection from HFD-induced obesity and hyperglycemia (Klöckener et al., 2011). Therefore, one could speculate that within the context of LepRb-deficiency, additional hypothalamic PTP1B deletion could sensitize VMH insulin signaling, leading to further metabolic impairment. Specifically how hypothalamic PTP1B deletion could exacerbate impaired glucose homeostasis within the context of LepRb deficiency remains unknown.

Taken together, these findings indicate that hypothalamic PTP1B deficiency can elicit metabolic improvements in body weight and adiposity within the context of diet-induced obesity where leptin resistance accumulates over time. In contrast, under the conditions of genetic leptin resistance where intracellular leptin signaling is completely impaired, PTP1B deficiency is ineffective at improving metabolic outcomes, demonstrating that within the hypothalamus, functional leptin receptor signaling is indeed required for PTP1B's metabolic effects. Based upon these findings, we propose two possible models of central PTP1B action in the control of energy homeostasis: (a) PTP1B's metabolic role in the CNS is exclusively as a negative regulator of leptin signaling, and the metabolic benefit of PTP1B deficiency is solely the result of enhanced leptin sensitivity, or (b) PTP1B acts on multiple signaling pathways (ex. insulin, non-leptin cytokines, growth factors etc.) including leptin, and the metabolic effects of PTP1B deficiency are a combination of numerous sensitized pathways of which leptin is the major contributor. Because PTP1B is a known negative regulator of insulin signaling (Seely et al., 1996; Bandyopadhyay et al., 1997; Byon et al., 1998) and JAK2 is associated with other non-leptin cytokine pathways implicated in the central control of energy balance, it seems the latter possibility is more likely, but the metabolic effects of these non-leptin pathways are masked by leptin's larger contribution. For example, though insulin and non-leptin cytokines including IL-6 and CNTF have been shown to have weight-reducing effects when administered centrally (Woods et al., 1979; Schwartz et al., 1992; McGowan et al., 1993; Sipols et al., 1995; Lambert et al., 2001; Wallenius et al., 2002a), their physiological regulation of energy homeostasis seems much less considerable when compared to leptin's role. Neuron-specific insulin receptor knockout mice only develop mild obesity (Brüning et al., 2000), and global IL-6 knockout mice display an obese phenotype only later in life (Wallenius et al., 2002b). In conjunction with our findings here, these studies further suggest that the leptin contribution towards energy homeostasis regulation is far more substantial than non-leptin pathways. Therefore, within the context of central PTP1B deficiency models,

sensitized leptin signaling likely underlies the majority of the lean metabolic phenotypes observed.

Alternatively, the lack of any phenotypic difference between Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ and Nkx2.1-LepRb$^{-/-}$ mice could be attributed to loss of leptin signaling during early development resulting in disrupted hypothalamic circuitry. Leptin has been demonstrated to act as a trophic factor and is required during neonatal brain development for formation of projections from the arcuate nucleus (Bouret and Simerly, 2004; Bouret et al., 2004). Thus, perhaps disrupted hypothalamic connectivity due to early loss of LepRb in Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice prevents any non-leptin pathway sensitization to elicit measurable, beneficial metabolic effects. Future studies utilizing inducible Cre lines may help to distinguish any confounding effects of early hypothalamic development on energy balance function. All in all, these findings highlight the continued importance of central leptin resistance as a main promoter of obesity.

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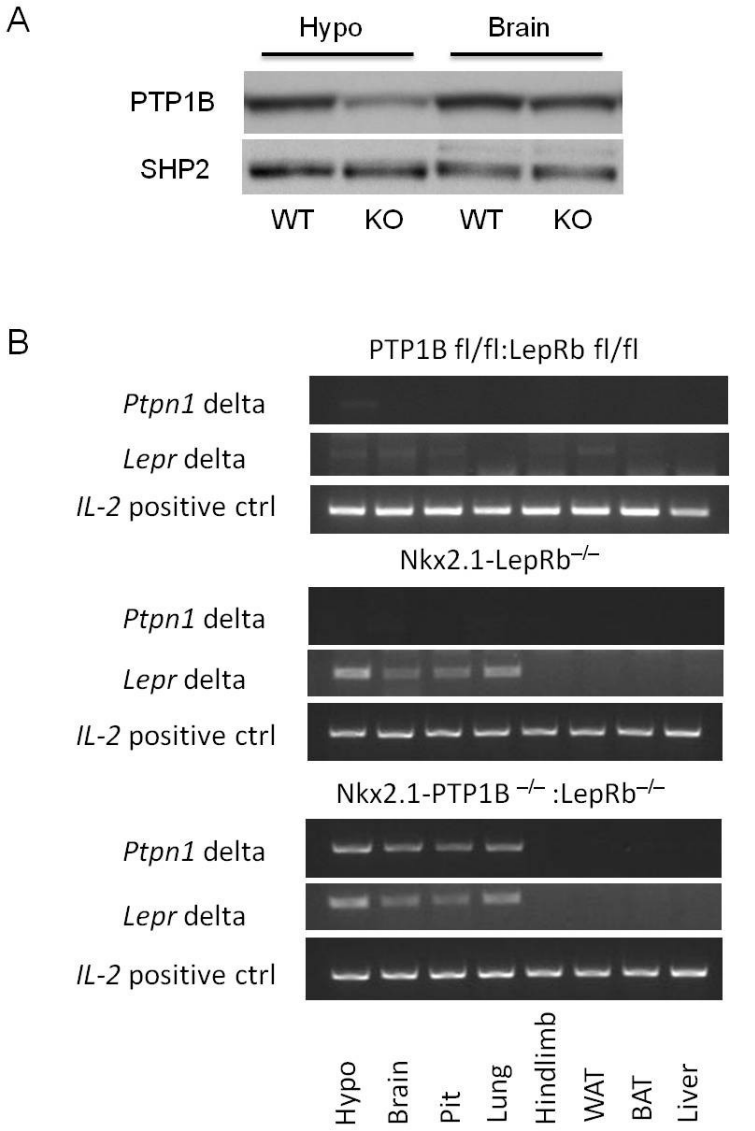


Figure 3.1 Detection of PTP1B deletion in Nkx2.1-PTP1B deficient mouse models. (A) PTP1B protein levels in the hypothalamus and brain of Nkx2.1-PTP1B$^{-/-}$ (KO) mice compared with PTP1B fl/fl controls (WT). SHP2 protein levels are shown as a loading control. (B) Detection of deletion of PTP1B or LepRb floxed alleles in PTP1B fl/fl:LepRb fl/fl, Nkx2.1-LepRb$^{-/-}$, and Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice. DNA was isolated from different tissues [hypothalamus (Hypo), extrahypothalamic brain, pituitary (Pit), lung, hindlimb, perigonadal white adipose tissue (WAT), brown adipose (BAT), and liver], and deletion of floxed allele was detected by PCR.

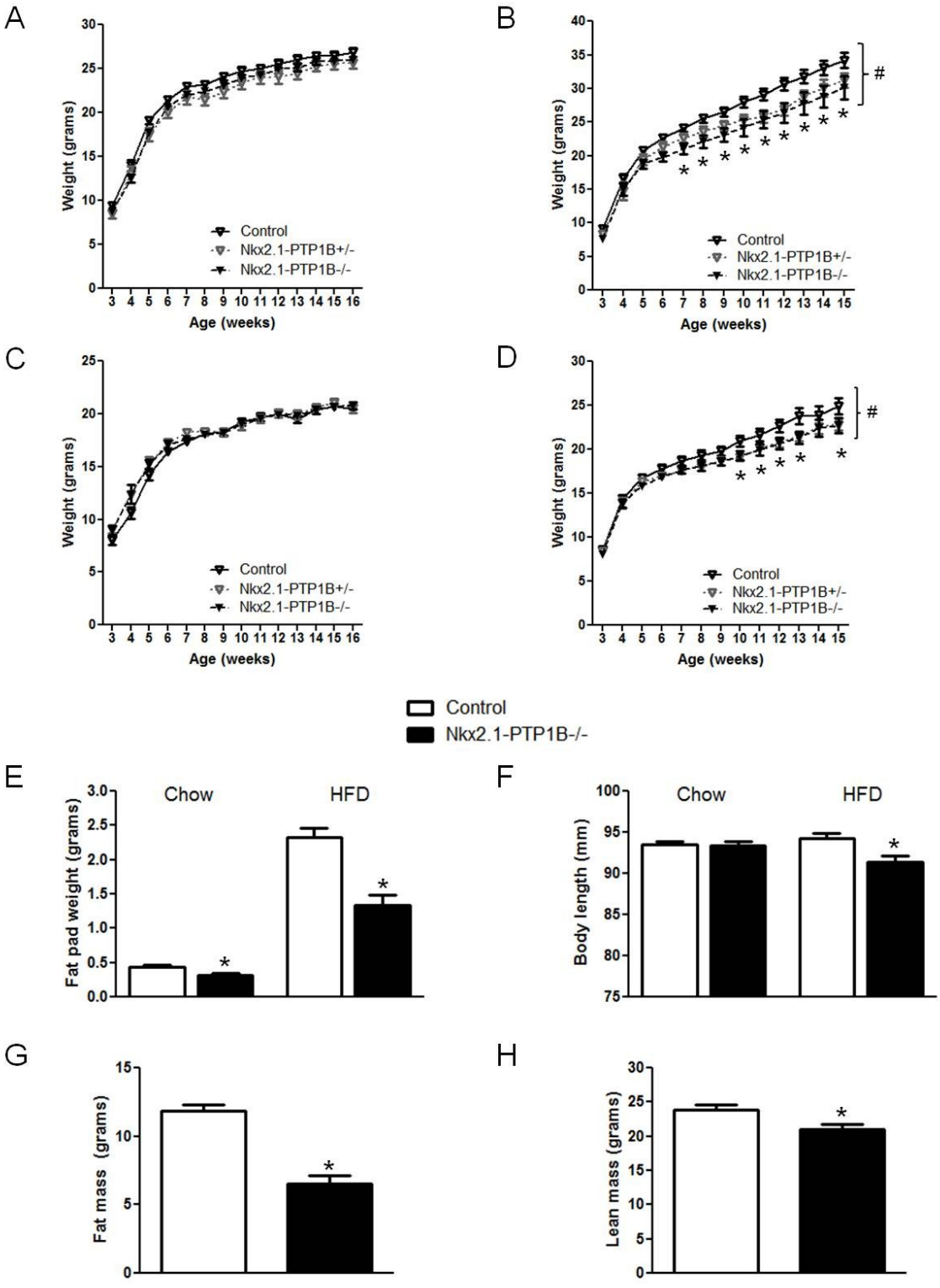


Figure 3.2 Nkx2.1-PTP1B$^{-/-}$ mice have reduced body weight and adiposity on HFD.

Figure 3.2 cont. (A) Body weights of male Nkx2.1-PTP1B$^{-/-}$ (n=9), Nkx2.1-PTP1B$^{+/-}$ (n=8), and control PTP1B fl/fl (n=14) mice on chow. (B) Body weights of male Nkx2.1-PTP1B$^{-/-}$ (n=6), Nkx2.1-PTP1B$^{+/-}$ (n=6), and control PTP1B fl/fl (n=17) mice on HFD. (C) Body weights of female Nkx2.1-PTP1B$^{-/-}$ (n=10), Nkx2.1-PTP1B$^{+/-}$ (n=8), and control PTP1B fl/fl (n=9) mice on chow. (D) Body weights of female Nkx2.1-PTP1B$^{-/-}$ (n=15), Nkx2.1-PTP1B$^{+/-}$ (n=11), and control PTP1B fl/fl (n=17) mice on HFD. (E) Epididymal fat pad weight for male Nkx2.1-PTP1B$^{-/-}$ (n=9 on chow, 6 on HFD) and control PTP1B fl/fl (n=14 on chow, 9 on HFD) mice on chow or HFD. (F) Body length for male Nkx2.1-PTP1B$^{-/-}$ (n=8 on chow, 6 on HFD) and control PTP1B fl/fl (n=14 on chow, 9 on HFD) mice on chow or HFD. (G) Fat mass as determined by NMR of male Nkx2.1-PTP1B$^{-/-}$ (n=6) and control PTP1B fl/fl (n=9) mice on HFD. (H) Lean mass as determined by NMR of male Nkx2.1-PTP1B$^{-/-}$ (n=6) and control PTP1B fl/fl (n=9) mice on HFD. All values are mean±SEM. Weight curves analyzed by two-way ANOVA with repeated measures: #p≤0.05. Fishers LSD post hoc pairwise comparisons between Nkx2.1-PTP1B$^{-/-}$ and wildtype controls:*p<0.05. Body composition and body length data analyzed by two tailed Student's t-test: *p<0.05.

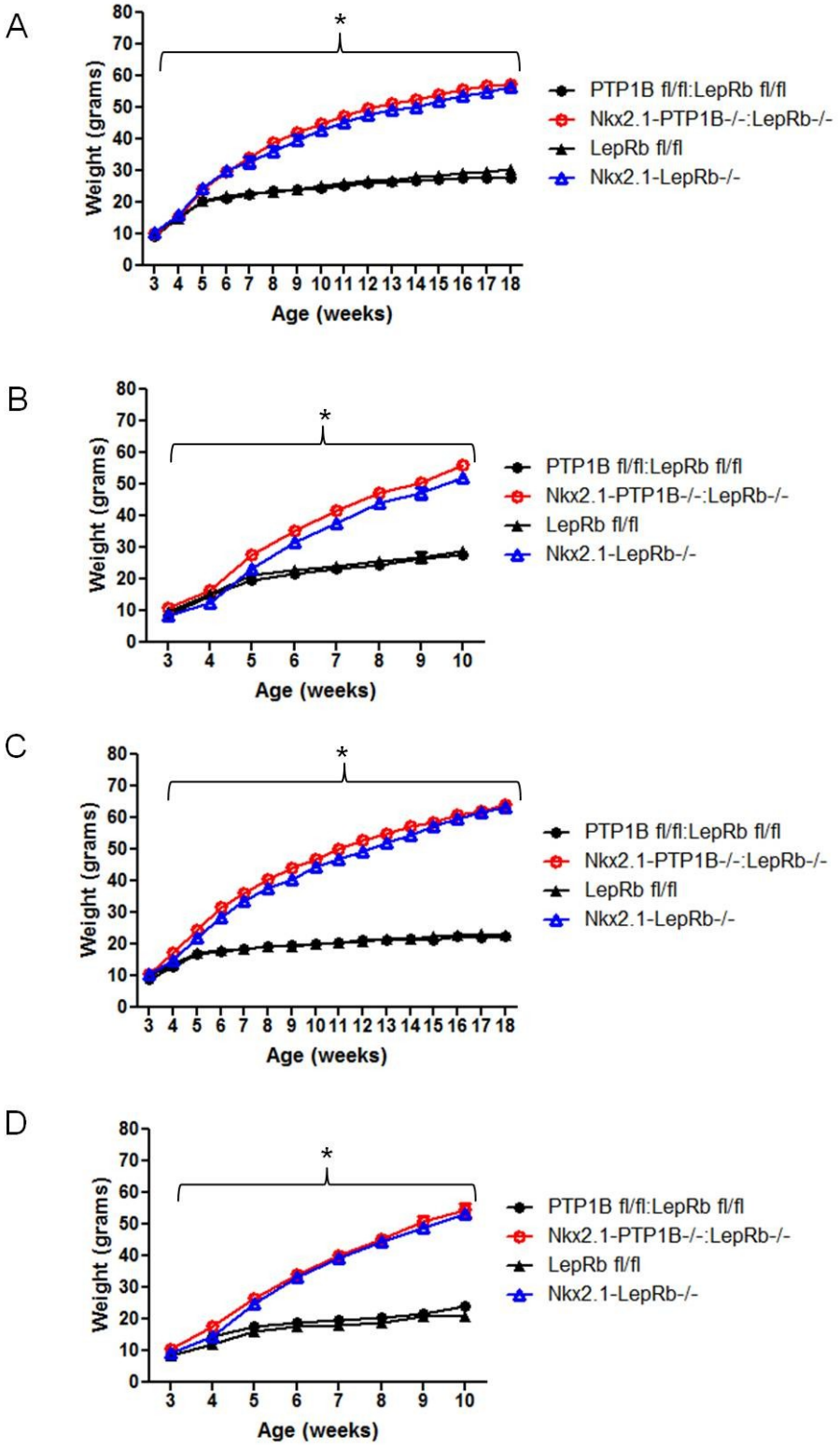


Figure 3.3 Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice show no difference in body weight compared to Nkx2.1-LepRb$^{-/-}$ mice.

Figure 3.3 cont. (A) Body weights of male Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ (n=8), Nkx2.1-LepRb$^{-/-}$ (n=9), control PTP1B fl/fl:LepRb fl/fl (n=14) and control LepRb fl/fl (n=9) mice on chow. (B) Body weights of male Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ (n=5), Nkx2.1-LepRb$^{-/-}$ (n=7), control PTP1B fl/fl:LepRb fl/fl (n=11) and control LepRb fl/fl (n=7) mice on HFD. (C) Body weights of female Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ (n=13), Nkx2.1-LepRb$^{-/-}$ (n=13), control PTP1B fl/fl:LepRb fl/fl (n=15) and control LepRb fl/fl (n=15) mice on chow. (D) Body weights of female Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ (n=6), Nkx2.1-LepRb$^{-/-}$ (n=13), control PTP1B fl/fl:LepRb fl/fl (n=6) and control LepRb fl/fl (n=5) mice on HFD. All values are mean±SEM. Weight curves analyzed by two-way ANOVA with repeated measures: *p<0.05.

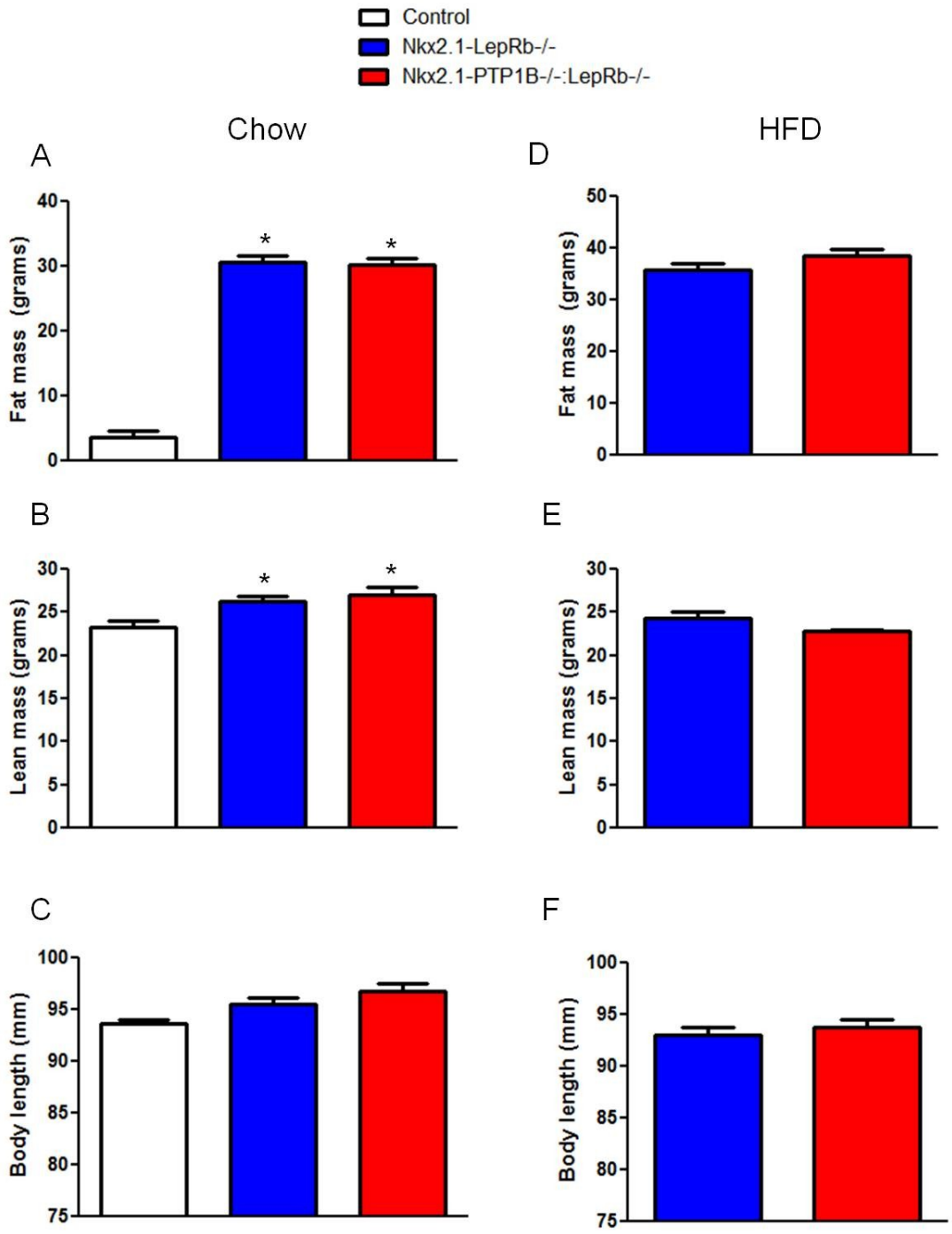


Figure 3.4 Male Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice show no difference in body composition compared to Nkx2.1-LepRb$^{-/-}$ mice. Fat mass (A) and lean mass (B) as determined by NMR of Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ (n=8), Nkx2.1-LepRb$^{-/-}$ (n=8), and control PTP1B fl/fl:LepRb fl/fl (n=3) mice on chow. (C) Body length of Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ (n=8), Nkx2.1-LepRb$^{-/-}$ (n=8), and control PTP1B fl/fl:LepRb fl/fl (n=3) mice on chow.

Figure 3.4 cont. Fat mass (D) and lean mass (E) as determined by NMR of Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ (n=5) and Nkx2.1-LepRb$^{-/-}$ (n=6) mice on HFD. (F) Body length of Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ (n=5) and Nkx2.1-LepRb$^{-/-}$ (n=6) mice on HFD. Body composition and body length on chow analyzed by one-way ANOVA followed by Student-Newman-Keuls pairwise comparison: *p<0.05 indicated group vs control.

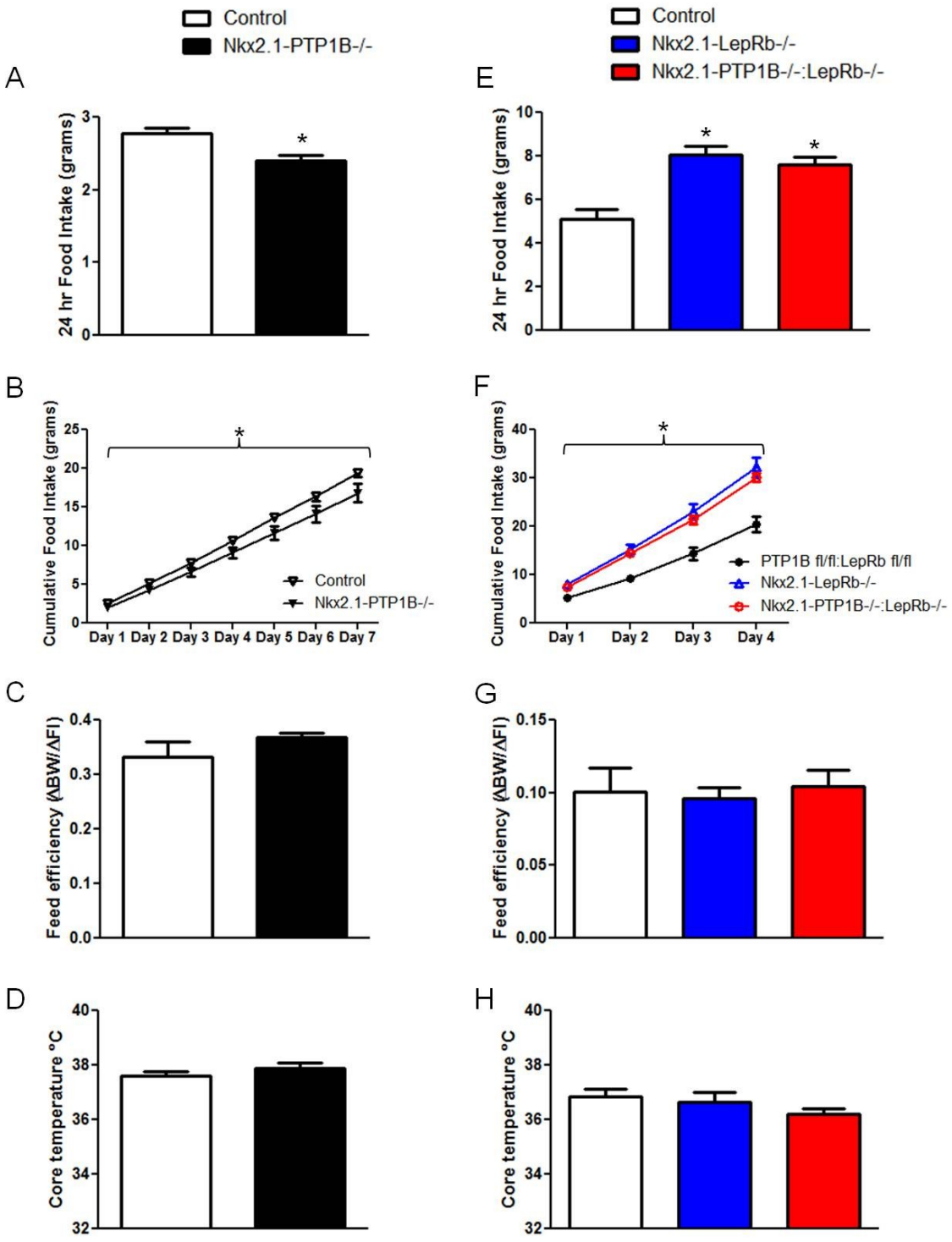


Figure 3.5 Nkx2.1-PTP1B$^{-/-}$ mice have decreased food intake on HFD whereas Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice show similar hyperphagia as Nkx2.1-LepRb$^{-/-}$ mice.

Figure 3.5 cont. Average daily (A) and cumulative (B) food intake of male Nkx2.1-PTP1B$^{-/-}$ (n=5) and control PTP1B fl/fl (n=6) on HFD. (C) 7 day feed efficiency of male Nkx2.1-PTP1B$^{-/-}$ (n=5) and control PTP1B fl/fl (n=6) on HFD. (D) Core temperature of male Nkx2.1-PTP1B$^{-/-}$ (n=5) and control PTP1B fl/fl (n=14) on HFD at 8 weeks of age. Average daily (E) and cumulative (F) food intake of male and female Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ (n=9), Nkx2.1-LepRb$^{-/-}$ (n=6), and control PTP1B fl/fl:LepRb fl/fl (n=6) mice on chow. (G) 4 day feed efficiency of male Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ (n=4), Nkx2.1-LepRb$^{-/-}$ (n=2), and control PTP1B fl/fl:LepRb fl/fl (n=3) mice on chow. (H) Core temperature of male Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ (n=4), Nkx2.1-LepRb$^{-/-}$ (n=2), control PTP1B fl/fl:LepRb fl/fl (n=8) mice on chow at 14 weeks of age. 24 hour food intake analyzed by two tailed Student's t-test or one-way ANOVA followed by Student-Newman-Keuls pairwise comparison: *p<0.05 indicated group vs. control. Cumulative food intake analyzed by two-way ANOVA with repeated measures: *p<0.05.

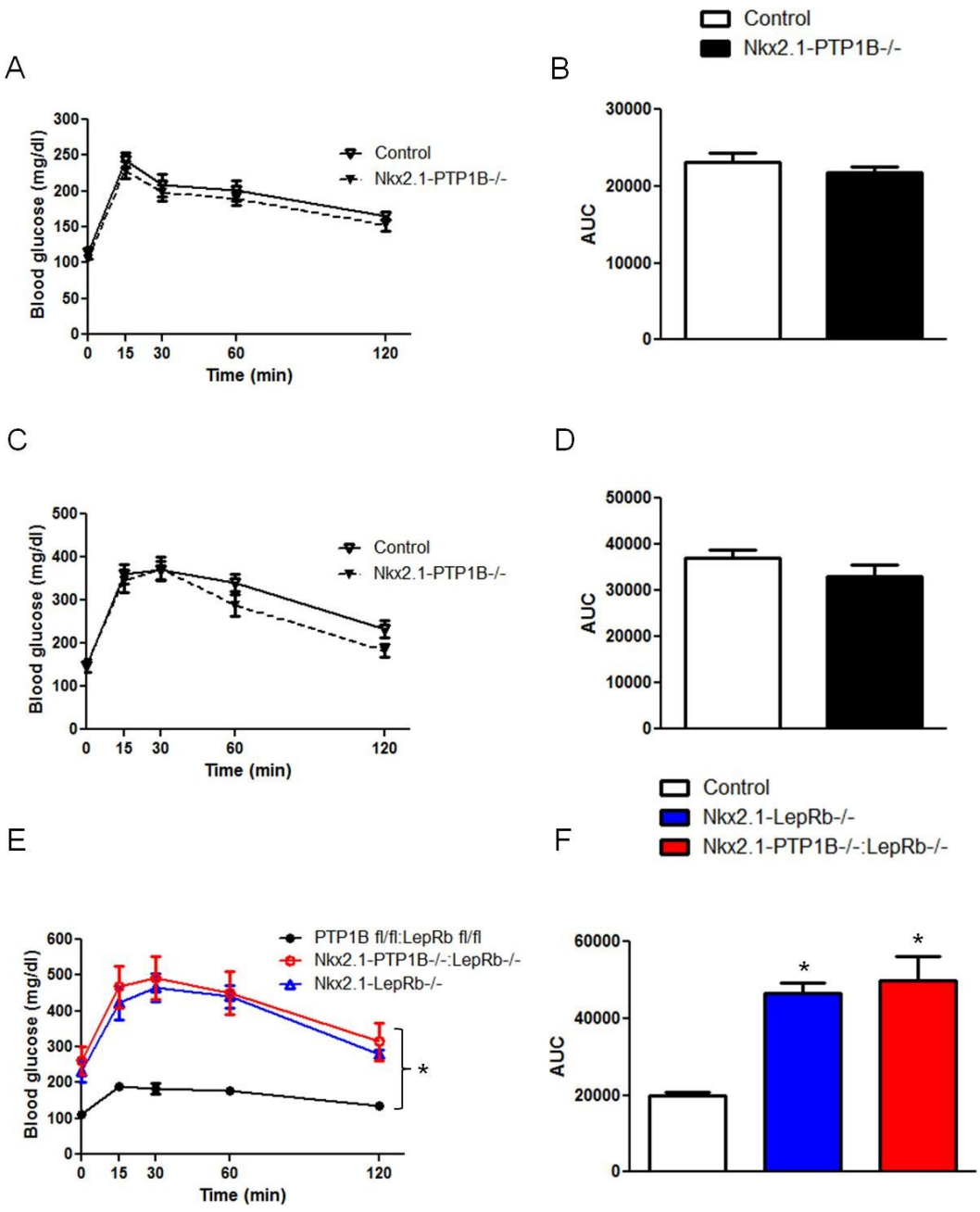


Figure 3.6 Glucose tolerance is unchanged in Nkx2.1-PTP1B deficient models compared to controls. (A) GTT for male Nkx2.1-PTP1B$^{-/-}$ (n=9) and control PTP1B fl/fl (n=8) mice on chow at 11 weeks of age. (B) Area under the curve (AUC) for blood glucose during GTT of male chow cohort. (C) GTT for male Nkx2.1-PTP1B$^{-/-}$ (n=6) and control PTP1B fl/fl (n=9) mice on HFD at 17 weeks of age. (D) AUC for blood glucose during GTT of male HFD cohort. (E) GTT for female PTP1B$^{-/-}$:LepRb$^{-/-}$ (n=5), Nkx2.1-LepRb$^{-/-}$ (n=7), and control PTP1B fl/fl:LepRb fl/fl (n=6) on chow at 13 weeks of age. (F) AUC for blood glucose during GTT of female chow cohort. GTT analyzed by two-way ANOVA with repeated measures: *p<0.05.

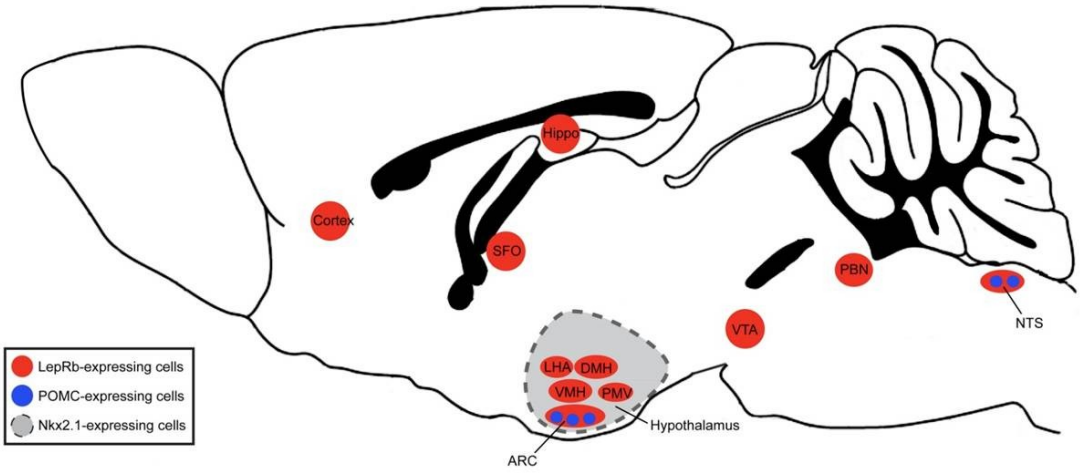
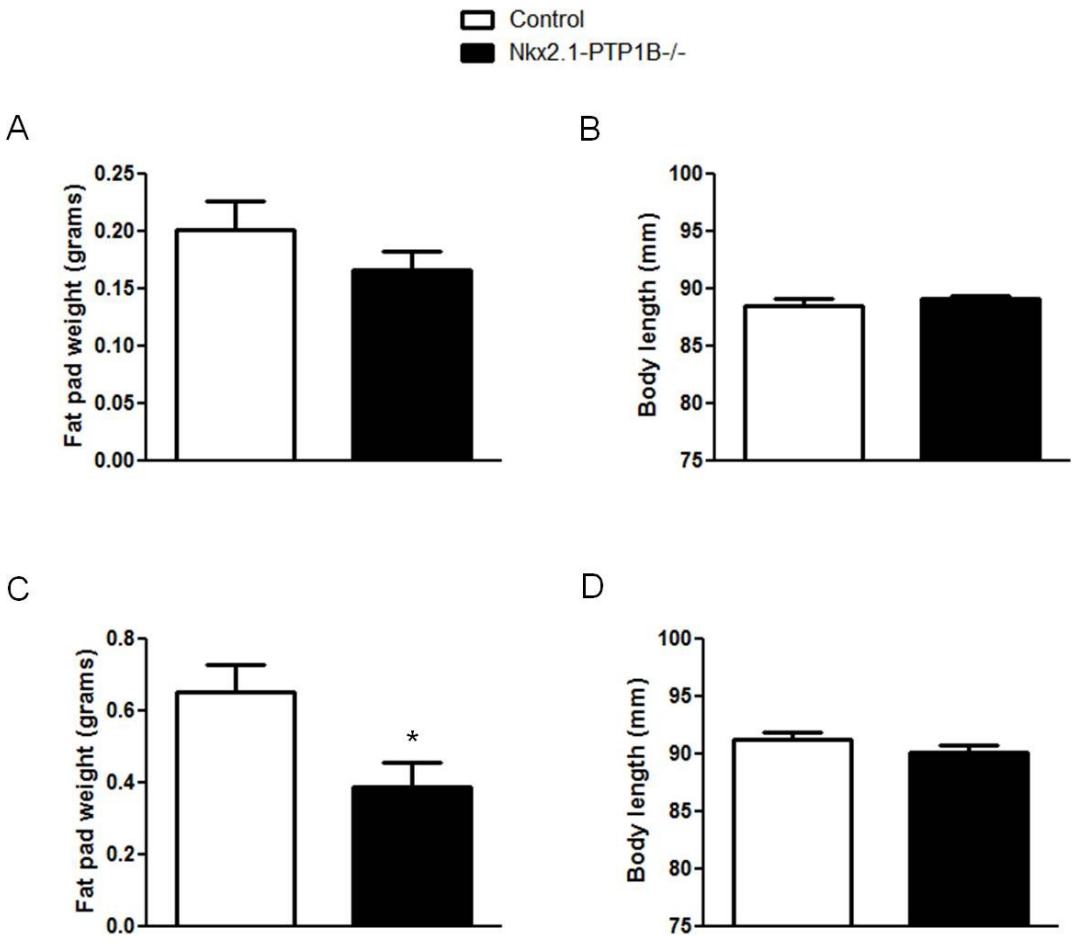
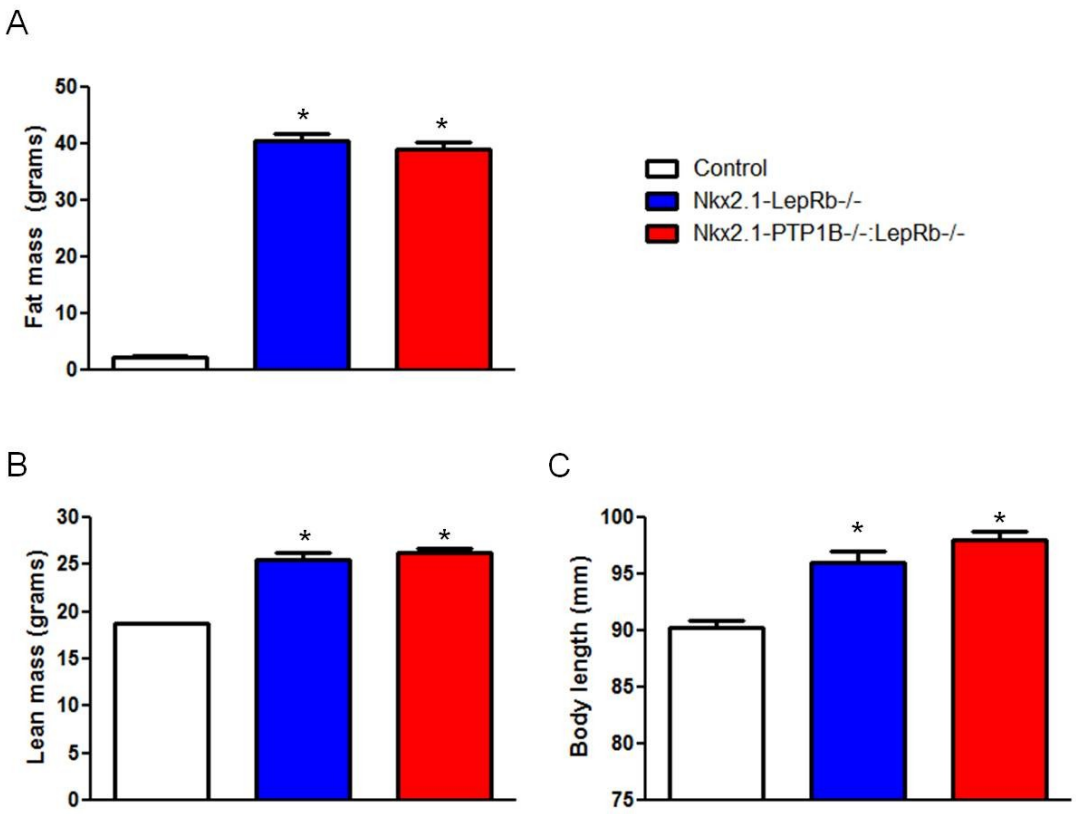


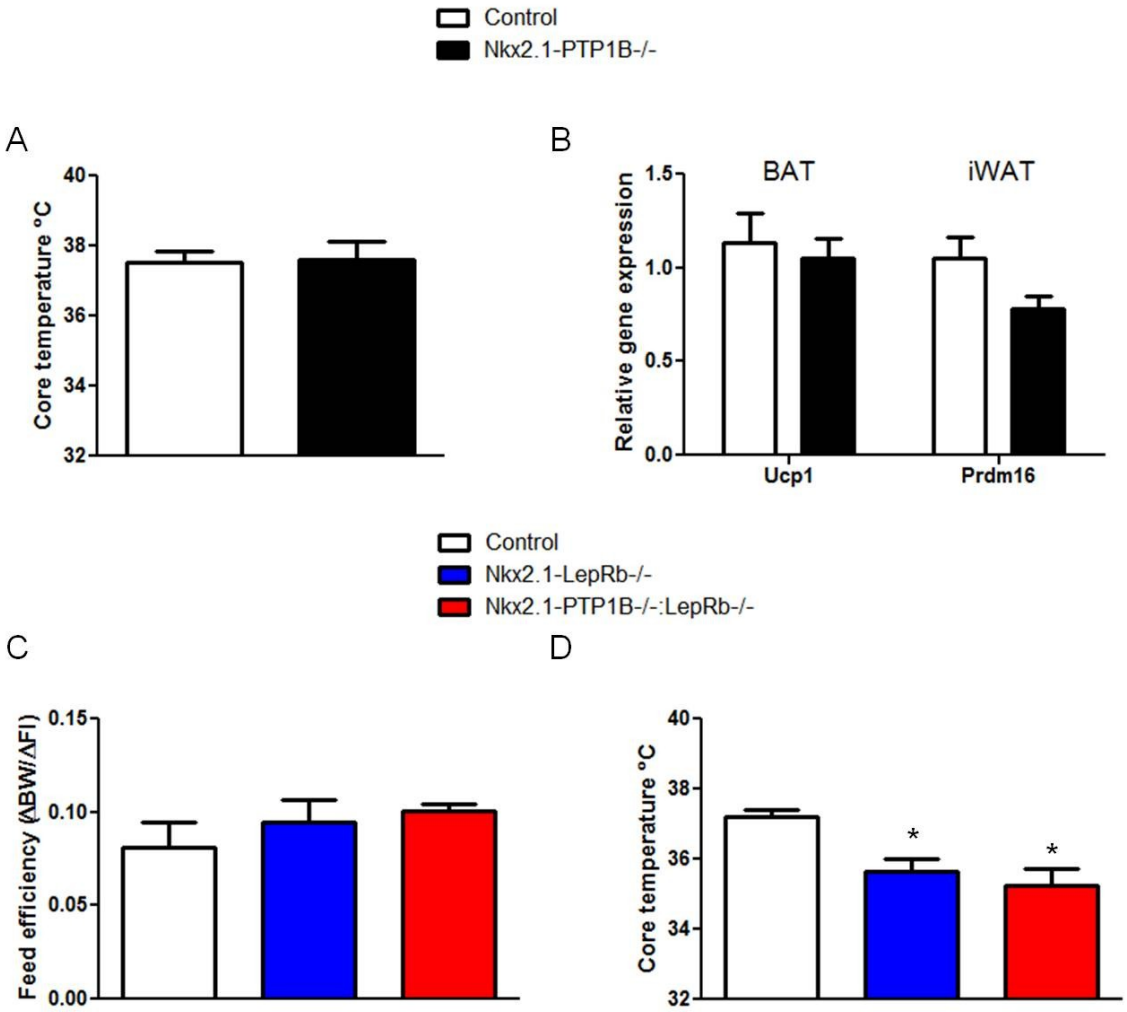
Figure 3.7 Comparison of the anatomic specificity of various CNS PTP1B deficient mouse models. LepRb and POMC are highly expressed in the arcuate nucleus (ARC) of the hypothalamus and are also found in the nucleus of the solitary tract (NTS) of the medulla. Outside of the NTS and hypothalamic nuclei including the ventromedial hypothalamic nucleus (VMH), dorsomedial hypothalamic nucleus (DMH), lateral hypothalamic area (LHA), and ventral premamillary nucleus (PMV), LepRb-expressing neurons are distributed broadly throughout the brain, with known extrahypothalamic sites including the cortex, hippocampus (Hippo), subfornical organ (SFO), ventral tegmental area (VTA), and the parabrachial nucleus (PBN).



Supplemental Figure 3.1 Female Nkx2.1-PTP1B$^{-/-}$ mice have reduced adiposity on HFD. (A) Epigonadal fat pad weight for Nkx2.1-PTP1B$^{-/-}$ (n=10) and control PTP1B fl/fl (n=8) mice on chow. (B) Body length for Nkx2.1-PTP1B$^{-/-}$ (n=9) and control PTP1B fl/fl (n=8) mice on chow. (C) Epigonadal fat pad weight for Nkx2.1-PTP1B$^{-/-}$ (n=9) and control PTP1B fl/fl (n=13) mice on HFD. (D) Body length for Nkx2.1-PTP1B$^{-/-}$ (n=9) and control PTP1B fl/fl (n=13) mice on HFD. Body composition and length data analyzed by two tailed Student's t-test: *p<0.05.



Supplemental Figure 3.2 Female Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice show no difference in body composition or body length compared to Nkx2.1-LepRb$^{-/-}$ mice on chow. Fat mass (A) and lean mass (B) as determined by NMR of Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ (n=9), Nkx2.1-LepRb$^{-/-}$ (n=7), and control PTP1B fl/fl:LepRb fl/fl (n=3) mice on chow. (C) Body length for Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ (n=13), Nkx2.1-LepRb$^{-/-}$ (n=8), and control PTP1B fl/fl:LepRb fl/fl (n=8) mice on chow. Body composition and body length analyzed by one-way ANOVA followed by Student-Newman-Keuls pairwise comparison: *p<0.05 indicated group vs. control.



Supplemental Figure 3.3 Nkx2.1-PTP1B$^{-/-}$ mice show no difference in core temperature or brown adipose tissue gene expression. (A) Core temperature of male Nkx2.1-PTP1B$^{-/-}$ (n=6) and control PTP1B fl/fl (n=9) on HFD at 17 weeks of age. (B) Expression of *Ucp1* and *Prdm16* in interscapular BAT and inguinal WAT, respectively of male mice Nkx2.1-PTP1B$^{-/-}$ (n=6) and control PTP1B fl/fl (n=9) on HFD. Female Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice show no difference in feed efficiency or core temperature compared to Nkx2.1-LepRb$^{-/-}$ mice on chow. (C) Feed efficiency of female Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ (n=5), Nkx2.1-LepRb$^{-/-}$ (n=4), and control PTP1B fl/fl:LepRb fl/fl (n=3) mice on chow. (D) Core temperature of female Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ (n=5), Nkx2.1-LepRb$^{-/-}$ (n=7), and control PTP1B fl/fl:LepRb fl/fl (n=6) mice on chow. Core temperature analyzed by one-way ANOVA followed by Student-Newman-Keuls pairwise comparison: *p<0.05 indicated group vs. control.

	Chow			HFD			Chow			HFD		
Genotype	**1B fl/fl**	**Nkx2.1-PTP1B+/-**	**Nkx2.1-PTP1B-/-**	**1B fl/fl**	**Nkx2.1-PTP1B+/-**	**Nkx2.1-PTP1B-/-**	**1B fl/fl:LepRb fl/fl**	**Nkx2.1-LepRb-/-**	**Nkx2.1-PTP1B-/-:LepRb-/-**	**1B fl/fl:LepRb fl/fl**	**Nkx2.1-LepRb-/-**	**Nkx2.1-PTP1B-/-:LepRb-/-**
Serum leptin (ng/ml)	1.65 ±0.21	1.14 ±0.18	1.20 ±0.16	23.12 ±4.61	6.69 ±1.38*	11.50 ±3.02*	8.18 ±0.77	90.34 ±4.79*	89.47 ±5.88*	16.19 ±4.91	104.68 ±3.74*	127.88 ±2.52*#
Corticosterone (ng/ml)	89.68 ±20.25	nd	106.59 ±28.96	62.35 ±24.53	nd	82.89 ±11.36	151.55 ±29.95	407.29 ±53.43*	361.75 ±95.75*	nd	nd	nd
Fasting blood glucose (mg/dl)	83.4 ±5.8	75.5 ±7.5	73.8 ±6.1	105.3 ±9.8	92.0 ±10.9	91.0 ±10.2	62.3 ±5.6	89.1 ±18.4	107.6 ±12.3*	94.3 ±9.2	219.1 ±29.3*	245.7 ±78.2*
Fasting serum insulin (ng/ml)	0.41 ±0.03	0.35 ±0.03	0.34 ±0.02	0.93 ±0.17	0.37 ±0.12*	0.55 ±0.06^	0.48 ±0.07	9.40 ±1.47*	14.43 ±2.76*#	0.62 ±0.11	13.25 ±4.37*	14.78 ±9.60*

Table 3.1 Metabolic and neuroendocrine parameters. Measures of fasting blood glucose and fasting serum insulin are from overnight fasted male animals at 12-14 weeks of age for controls, Nkx2.1-PTP1B$^{+/-}$, Nkx2.1-PTP1B$^{-/-}$ on chow or HFD. Fasting blood glucose and fasting serum insulin of Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$, Nkx2.1-LepRb$^{-/-}$, and control PTP1B fl/fl:LepRb fl/fl mice are taken at 12-14 weeks on chow and at 9 weeks on HFD. Measures of serum leptin and corticosterone are from *ad lib* fed male animals at 7 weeks of age. Fasting blood glucose and serum measures analyzed by one-way ANOVA followed by Fisher's LSD pairwise comparison: *p<0.05, ^p=0.08 indicated group vs. control. #p<0.05 Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ vs. Nkx2.1-LepRb$^{-/-}$. nd, not determined.

CHAPTER 4: IL-6 JAK/STAT Signaling is not regulated by Endogenous PTP1B in a Hypothalamic Cell Line

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Abstract

Protein tyrosine phosphatase 1B (PTP1B) is a ubiquitously expressed tyrosine phosphatase and a known negative regulator of leptin signaling via the direct dephosphorylation of janus kinase 2 (JAK2). Interleukin-6 (IL-6) is an inflammatory cytokine which has been implicated in the central control of energy balance, and like leptin, IL-6 signals through a type I cytokine receptor pathway involving janus kinase activation. Using an immortalized, mouse embryonic hypothalamic cell line (mHypoE-N46), we examined whether PTP1B can regulate IL-6 induced JAK/STAT signaling. We determined that IL-6 dose-dependently induces STAT3 phosphorylation in mHypoE-N 46 cells, but does not detectably induce JAK2 or TYK2 phosphorylation. Knocking down endogenous PTP1B using viral mediated shRNA does not enhance IL-6 induced STAT3 phosphorylation. On the other hand, PTP1B overexpression suppresses IL-6 induced STAT3 phosphorylation, suggesting PTP1B may regulate IL-6 signaling in a unidirectional and JAK-independent manner. Overall, these results suggest that endogenous PTP1B is likely not a key regulator of hypothalamic IL-6 signaling. However, it remains possible that under conditions of elevated hypothalamic PTP1B expression (as seen in high-fat diet and obesity), IL-6 activation of STAT3 may be impaired.

Introduction

Numerous cellular signaling pathways in the CNS (central nervous system) have been shown to play a role in the control of body weight. Many of these pathways are regulated by tyrosine phosphorylation, and protein tyrosine phosphatases have been implicated in the central regulation of energy homeostasis. Protein tyrosine phosphatase 1B (PTP1B) is a ubiquitously expressed tyrosine phosphatase and a known negative regulator of leptin signaling (Cheng et al., 2002; Zabolotny et al., 2002). When leptin binds to its receptor, LepRb, a conformational change results in activation of the associated tyrosine kinase janus kinase 2 (JAK2). JAK2 autophosphorylates and phosphorylates several tyrosine residues along the cytoplasmic tail of LepRb allowing for downstream signal transduction, including the recruitment of (signal transducer and activator of transduction 3) STAT3, a transcription factor known to mediate leptin's effects on energy balance by regulating neuropeptide gene expression. PTP1B negatively regulates leptin signaling through the direct dephosphorylation of JAK2 (Myers et al., 2001; Cheng et al., 2002; Zabolotny et al., 2002), and its role in the central control of energy balance has been clearly demonstrated through the generation of PTP1B-deficient mouse models. Whole body, whole brain, LepRb-expressing cell-, or POMC-specific PTP1B-/- mice are resistant to diet-induced weight gain, likely due to enhanced leptin sensitivity (Elchebly et al., 1999; Klaman et al., 2000; Bence et al., 2006; Banno et al., 2010; Tsou et al., 2012). However, leptin deficient *ob/ob*:PTP1B-/- double mutants show attenuated weight gain compared to *ob/ob* single mutants, suggesting that PTP1B may also regulate non-leptin pathways implicated in the CNS control of energy balance (Cheng et al., 2002). Interleukin-6 (IL-6) is a pro- or anti-inflammatory cytokine, depending on cellular context, which has been shown to mediate a variety of physiological functions including induction of acute phase proteins, cell proliferation, survival, and apoptosis (Hodge et al., 2005; Ropelle et al., 2010). Additionally, IL-6 has been demonstrated to have central effects on energy balance, and obesity has been attributed with a persistent, low dose inflammatory state (Dandona et al., 2004). IL-6-/- mice display mature onset obesity which is ameliorated with IL-6 replacement therapy (Wallenius et al., 2002b).

Furthermore, intracerebroventricular (ICV) but not intraperitoneal injection of IL-6 enhances energy expenditure in rats, and chronic ICV delivery over two weeks reduces food intake, body weight, and adiposity (Wallenius et al., 2002a, 2002b). Like leptin, IL-6 signals through type I cytokine receptors. When IL-6 binds to its receptor complex, a conformational change in the receptor complex leads to activation of an associated janus kinase: JAK1, JAK2, or TYK2. The activated JAK autophosphorylates and phosphorylates the intracellular tail of gp130Rβ, the transmembrane signaling subunit of the IL-6 receptor complex. This allows for downstream signal transduction and recruitment of STAT transcription factors such as STAT3 and STAT1 (Heinrich et al., 1998). Given IL-6 signaling, like leptin, involves downstream tyrosine phosphorylation events, phosphatase activity plays a role in negatively regulating IL-6-induced signaling. Indeed, PTP1B has been demonstrated as a negative regulator of IL-6-stimulated STAT3 phosphorylation in smooth muscle cells and hepatocytes (Chang, 2011; Revuelta-Cervantes et al., 2011). Importantly, whether or not PTP1B can regulate IL-6-induced JAK-STAT signaling in the CNS is still unknown. Here, we use an immortalized, mouse embryonic hypothalamic cell line (mHypoE-N46) to examine whether PTP1B plays a role in IL-6-induced JAK-STAT signaling.

Materials and Methods

Cell culture and reagents. mHypoE-N46 cells were grown in a monolayer and maintained in DMEM plus 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Platinum-E (Plat-E) packaging cell lines were maintained in DMEM plus 10% FBS, 1% penicillin/streptomycin, 1ug/ml puromycin and 10ug/ml blasticidin. Recombinant mouse IL-6 was obtained from BioLegend (San Diego, CA). Recombinant mouse leptin was obtained from A.F. Parlow, NHPP (Torrance, CA). Mouse PTP1B antibody was homemade (Klaman et al., 2000). pSTAT3, pJAK2, and total JAK2 antibodies were purchased from Cell Signaling (Danvers, MA). pTYK2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Viral mediated transduction of PTP1B shRNA or PTP1B overexpression vectors. PTP1B knockdown was achieved using shRNA constructs designed against mouse PTP1B using the pSUPER.retro.puro vector system (OlgoEngine). Human PTP1B overexpression was achieved through the use of pWZL-hygro vector backbone. Knockdown or overexpression vectors were transfected using PEI into Plat-E packaging cells. 48 hours later, viral supernatant was harvested, filtered, and stored at -80°C. For PTP1B knockdown or overexpression, mHypoE-N46 cells were grown to 50% confluency and infected with retroviral supernatant. 24 hours later, culture media was replaced with selection media (culture media plus puromycin 1.75ug/ml or hygromycin 450ug/ml). Kill curves were completed to determine the dose for antibiotic selection. Cells were maintained in selection media for 4-5 days prior to beginning of signaling studies.

Immunoblotting. mHypoE-N46 cells were serum starved overnight for 16 hours, and rinsed with serum free media prior to stimulation with 1-20ng/ml IL-6. At indicated time points, cells were harvested in modified RIPA buffer (10mM Tris pH 7.4, 150mM NaCl, 0.1% SDS, 1% Triton X-100, 1% Na deoxycholate, 5mM EDTA, 10mM Sodium Pyrophosphate, 10mM B-glycerophosphate, 50mM NaF) containing fresh protease (Protease inhibitor cocktail, Sigma) and phosphatase inhibitors (1mM sodium ortho-vanadate) and 20-40ug of protein were resolved by SDS-PAGE and transferred to PVDF membranes. Immunoblots were performed with PTP1B, pSTAT3, pJAK2, pTYK2, STAT3, and JAK2 antibodies.

Results

Leptin stimulation increases JAK2 and STAT3 phosphorylation in a hypothalamic cell line. mHypoE-N46 cells were previously characterized and were found to be leptin responsive (Mayer and Belsham, 2009; Dhillon and Belsham, 2011). Dhillon and Belsham found that 100nM leptin significantly induced JAK2 phosphorylation but not STAT3 phosphorylation in mHypoE-N46 cells serum starved for 4 hours. Here, we demonstrate that mHypoE-N46 cells respond to 100nM and 200nM leptin stimulation, inducing both JAK2 and STAT3 phosphorylation in mHypoE-N46 cells

serum starved overnight (Fig. 4.1A). Relative pJAK2 and pSTAT3 levels were increased in leptin-treated but not vehicle-treated mHypoE-N46 cells at 1 and 10 minute time points. These findings suggest mHypoE-N46 cells are a suitable model for studying intracellular JAK/STAT signaling.

IL-6 stimulation dose-dependently increases STAT3 but not JAK2 nor TYK2 phosphorylation in mHypoE-N46 cells.

To first determine whether or not mHypoE-N46 cells respond to exogenous IL-6, we conducted a dose-response signaling study. Serum starved mHypoE-N46 cells were treated with vehicle or 1, 5, 10, or 20 ng/ml IL-6 for 15 minutes, and relative levels of STAT3 phosphorylation were determined by Western blot analysis. IL-6 treatment dose-dependently induced STAT3 phosphorylation in mHypoE-N46 cells, whereas unstimulated or vehicle treated cells showed relatively low levels of pSTAT3 expression (Fig. 4.1B).

Because the janus kinases JAK2 and TYK2 are known direct substrates of PTP1B and have been implicated in mediating IL-6 signaling, we also looked at whether JAK2 or TYK2 phosphorylation was induced by IL-6 dose treatment. In contrast to leptin-treated cells, mHypoE-N46 cells treated with IL-6 did not show any changes in pJAK2 levels compared to vehicle treated cells. Additionally, levels of TYK2 phosphorylation were unchanged between vehicle and IL-6 treated cells, suggesting that JAK2 and TYK2 may not mediate intracellular IL-6-STAT3 signaling in mHypoE-N46 cells (Fig. 4.1B).

IL-6 exposure does not affect PTP1B expression in mHypoE-N46 cells.

Central IL-6 administration has been shown to downregulate hypothalamic PTP1B protein levels *in vivo* (Chiarreotto-Ropelle et al., 2013). Therefore, we looked at whether IL-6 exposure could affect PTP1B expression levels in mHypoE-N46 cells. Cells were incubated with vehicle or 5ng/ml IL-6 for 5, 30, 60, or 120 minutes, and PTP1B protein levels were measured by Western

blot analysis. We observed no difference in PTP1B expression levels between mHypoE-N46 cells exposed to vehicle or IL-6 at any time point over the course of 120 minutes (Fig. 4.1C).

PTP1B knockdown does not enhance IL-6 induced STAT3 phosphorylation in mHypoE-N46 cells.

To determine whether IL-6 stimulated JAK-STAT signaling is regulated by endogenous PTP1B, we knocked down (KD) PTP1B expression in mHypoE-N46 cells via retroviral delivery of a PTP1B shRNA construct. Cells were transduced with virus expressing either scramble control or PTP1B shRNA, followed by vehicle or IL-6 stimulation. IL-6 (1ng/ml) induced STAT3 phosphorylation in both scramble and PTP1B KD cells at 5, 15 and 30 minutes to a similar extent; STAT3 phosphorylation was not enhanced in PTP1B KD cultures (Fig. 4.2A).

PTP1B overexpression suppresses IL-6 induced STAT3 phosphorylation in mHypoE-N46 cells.

In addition to knocking down PTP1B expression, we examined the effects of PTP1B overexpression on IL-6 stimulated JAK-STAT signaling. Interestingly, whereas PTP1B KD had no effect on IL-6 induced STAT3 phosphorylation, overexpression of PTP1B suppressed IL-6-induced STAT3 phosphorylation (Fig. 4.2B). In a separate experiment, we also looked at whether JAK2 phosphorylation would be affected by human PTP1B overexpression in mHypoE-N46 cells. JAK2 phosphorylation was unaffected by IL-6 stimulation (Fig. 4.2C). Surprisingly, PTP1B overexpression did not affect JAK2 phosphorylation, though IL-6-induced STAT3 phosphorylation was suppressed (Fig. 4.2C).

Discussion

Whether or not PTP1B's central metabolic effects are mediated exclusively through leptin receptor signaling or through other pathways implicated in the CNS body weight control is unknown. Because IL-6 has been shown to have CNS-specific weight reducing effects and its

signaling pathway, like leptin's, is subject to regulation by tyrosine phosphorylation, we examined whether PTP1B could regulate hypothalamic IL-6 signaling *in vitro.* Our data demonstrate that IL-6 dose-dependently induces STAT3, but not JAK2 or TYK2, phosphorylation in mHypoE-N46 cells and that endogenous PTP1B does not appear to be a key regulator of IL-6 JAK-STAT signaling.

IL-6 has been shown to signal through the JAK-STAT signaling pathway (reviewed in Heinrich et al., 1998). Past studies have demonstrated JAK1, JAK2, and TYK2 are all potential mediators of IL-6-stimulated JAK-STAT signaling (Berger et al., 1994; Lütticken et al., 1994; Narazaki et al., 1994). In mHypoE-N46 cells, IL-6 dose-dependently increased pSTAT3 levels but had no effect on either JAK2 or TYK2 phosphorylation. JAK1 has been shown to be necessary for activation of downstream STAT signaling whereas JAK2 and TYK2 were shown to be dispensable in human fibrosarcoma cell lines (Guschin et al., 1995). Therefore, in mHypoE-N46 cells JAK1 may be mediating STAT3 activation. Alternatively, JAK-independent activation of STAT3 may be occurring. In murine plasma B cells, IL-6 activation of STAT3 was shown to bypass all 3 janus kinases, suggesting that IL-6 JAK/STAT signaling is different depending upon cellular context (Kopantzev et al., 2002). Future studies are needed to address whether JAK1-mediated or JAK-independent activation of STAT3 occurs in this hypothalamic cell line, as well as in the hypothalamus *in vivo*.

Central IL-6 administration has been shown to downregulate hypothalamic PTP1B protein expression in obese rats after 6 hours (Chiarreotto-Ropelle et al., 2013). In myocytes, however, PTP1B gene expression and activity is increased with 24 hour IL-6 treatment (Nieto-Vazquez et al., 2008). We determined that IL-6 did not affect PTP1B expression in mHypoE-N46 cells after a 2 hour treatment. Further study with additional IL-6 doses and longer-term time points is required to conclusively determine whether IL-6 treatment regulates PTP1B expression in mHypoE-N46 cells.

IL-6-induced STAT3 activation has been demonstrated to be regulated by PTP1B in smooth muscle cells and hepatocytes (Chang, 2011; Revuelta-Cervantes et al., 2011). Neither study determined whether PTP1B regulated IL-6 JAK-STAT signaling via dephosphorylation of JAK2 or TYK2 but showed clear effects of PTP1B expression on IL-6 induced pSTAT3 levels. Consistent with the lack of IL-6 induced JAK2 and TYK2 phosphorylation, we show that PTP1B KD does not enhance STAT3 phosphorylation in mHypoE-N46 cells, indicating that endogenous PTP1B may not regulate IL-6 induced STAT3 phosphorylation. In PTP1B deficient macrophages isolated from myeloid-cell-specific PTP1B-/- mice, IL-6 induced STAT3 phosphorylation was not elevated (Grant et al., 2013), suggesting that PTP1B differentially regulates IL-6 signaling in different cell types. Alternatively, although PTP1B levels were significantly decreased by shRNA knockdown, the residual PTP1B activity may be sufficient to regulate IL-6 signaling in these cells.

Despite PTP1B KD having no effect on IL-6-induced STAT3 activation, overexpression of human PTP1B results in a complete suppression of IL-6-induced STAT3 phosphorylation; JAK2 phosphorylation remains unaffected by IL-6 treatment and also is unaffected by PTP1B overexpression. Additional studies are necessary to elucidate the mechanism by which PTP1B may regulate IL-6 induced STAT3 phosphorylation. Though JAK2 and TYK2 are known substrates of PTP1B within the JAK-STAT signaling pathway, there is evidence suggesting PTP1B may directly dephosphorylate STAT3 (Lund et al., 2005).

Taken together, the findings from the PTP1B KD and overexpression experiments suggest that endogenous PTP1B is not a key regulator of IL-6-stimulated JAK-STAT signaling in mHypoE-N46 cells. Thus, enhanced IL-6 signaling likely does not underlie the improved metabolic effects of PTP1B deficiency; however, given that increased PTP1B expression suppresses IL-6-induced STAT3 activation in mHypoE-N46 cells, IL-6 signaling could be impaired when hypothalamic PTP1B levels rise in the context of HFD-induced obesity or inflammation. Indeed, increased

hypothalamic PTP1B expression has been demonstrated in mice with HFD-feeding or tumor necrosis factor α (TNFα) administration (Zabolotny et al., 2008; White et al., 2009; Picardi et al., 2010; Loh et al., 2011; Ito et al., 2012), and any effects on IL-6 signaling could be studied in these models. Because different hypothalamic cell lines may exhibit different signaling capacities, the preceding experiments could be repeated in different hypothalamic cultures (ex. GT1-7's, other mHypoE lines), or *in vivo*, to further explore PTP1B's potential role as a regulator of central IL-6 signaling.

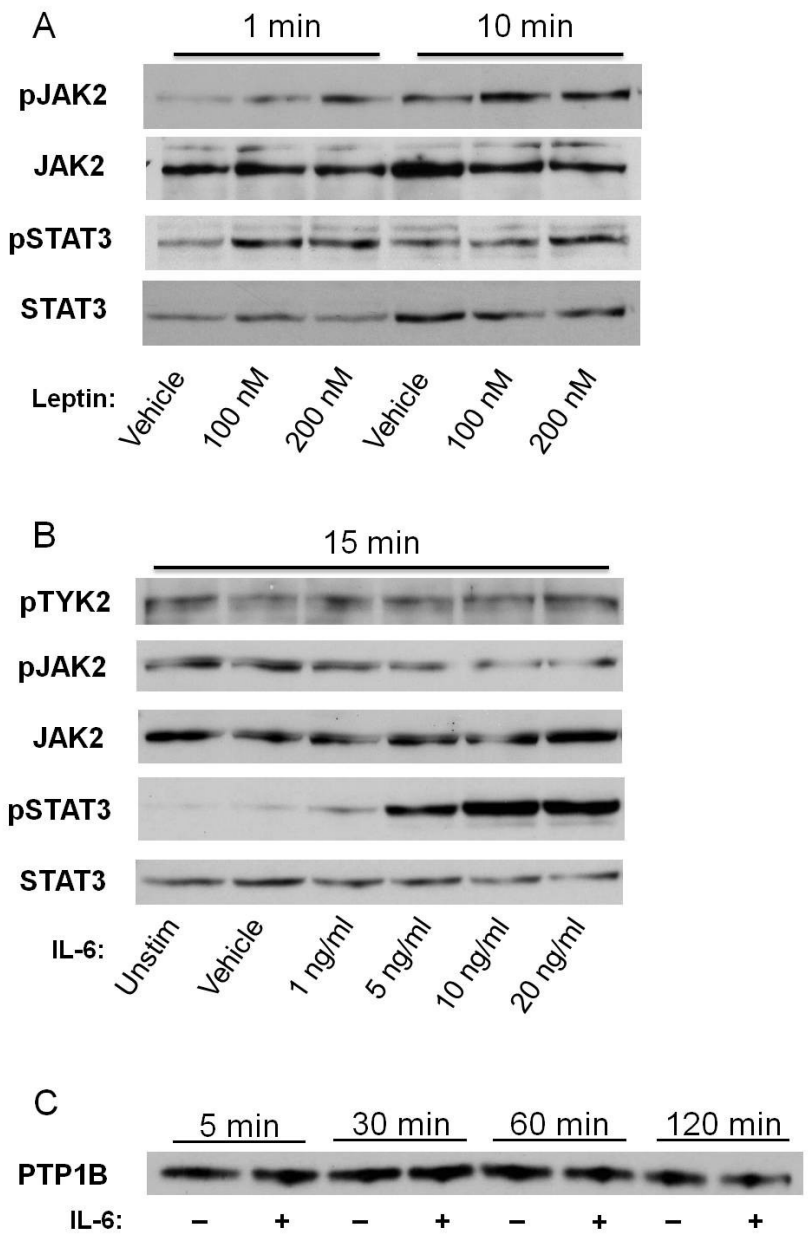

Figure 4.1 (A) Leptin induces JAK2 and STAT3 phosphorylation in mHypoE-N46 cells. mHypoE-N46 cells were stimulated with vehicle or leptin (100 nM or 200 nM) for 1 or 10 minutes. Protein was isolated and immunoblotted with anti-pJAK2, anti-JAK2, anti-pSTAT3, or anti-STAT3 antibodies. (B) IL-6 induces STAT3 phosphorylation independent of JAK2 or TYK2 activation. mHypoE-N46 cells were unstimulated or treated with vehicle or IL-6 (1, 5, 10, or 20 ng/ml) for 15 minutes. Protein was isolated and immunoblotted with anti-pTYK2, anti-pJAK2, anti-JAK2, anti-pSTAT3, or anti-STAT3 antibodies. (C) IL-6 treatment does not affect PTP1B expression. mHypoE-N46 cells were incubated with vehicle or 5ng/ml IL-6 for up to 2 hours. Protein was isolated and immunoblotted for PTP1B.

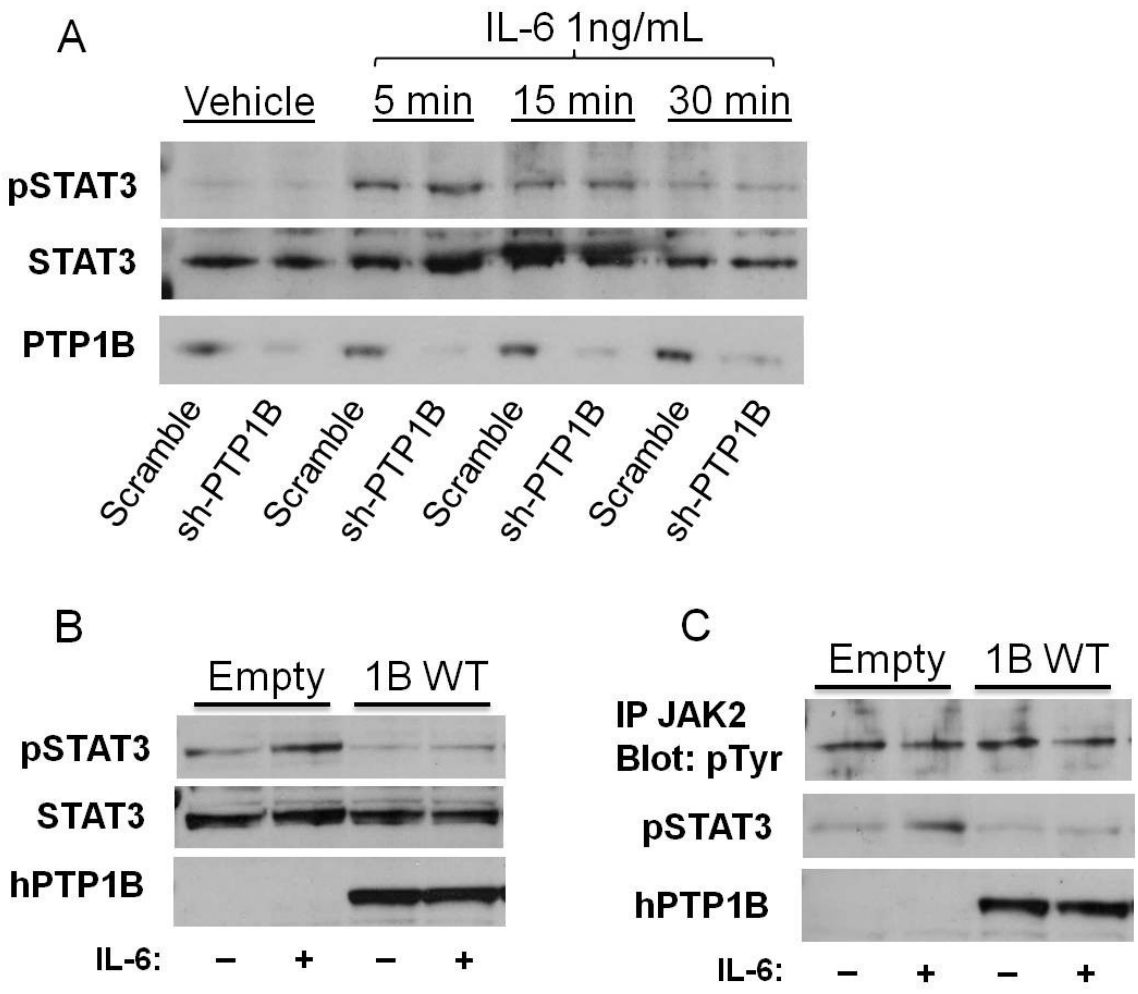

Figure 4.2 (A) PTP1B does not regulate IL-6-induced STAT3 activation in mHypoE-N46 cells. mHypoE-N46 cells transduced with scramble or PTP1B shRNA and treated with vehicle or IL-6 (1 ng/ml) for up to 30 minutes. Protein was isolated and immunoblotted for pSTAT3, STAT3, and PTP1B. (B) and (C) PTP1B overexpression suppresses IL-6-induced STAT3 phosphorylation. mHypoE-N46 cells were transduced with PTP1B overexpression constructs (empty, WT) and treated with vehicle or IL-6 (1 ng/ml) for 5 minutes. Protein was isolated and either immunoblotted for pSTAT3, STAT3, or human PTP1B or immunoprecipitated with anti-JAK2 and immunoblotted with anti-pTyr.

CHAPTER 5: Conclusions and Future Directions

PTP1B is a ubiquitously expressed protein tyrosine phosphatase shown to centrally regulate body weight and metabolism as demonstrated by a number of CNS-specific PTP1B-deficient mouse models. Though improved leptin sensitivity is the putative mechanism by which central PTP1B deletion confers protective body weight effects, whether or not PTP1B regulates other leptin-independent pathways implicated in the control of energy balance remains unclear. Here we studied the role of PTP1B and its interaction with leptin-dependent and leptin-independent signaling. In chapter 2, we demonstrated the extent to which the metabolic effects of PTP1B deletion are mediated within leptin receptor-expressing cells. LepRb-specific PTP1B-/- mice show an improved metabolic phenotype similar to that of whole body PTP1B-/- mice, indicating that PTP1B in LepRb-expressing cells plays a major role in the central control of energy balance and glucose homeostasis. Next in chapter 3, we showed that not only does PTP1B within the hypothalamus contribute to the central regulation of energy balance, but that extrahypothalamic PTP1B also plays a role, as the extent of the metabolic improvements of hypothalamic PTP1B-/- mice were relatively modest when qualitatively compared to that of whole brain or LepRb-specific PTP1B-/- mice. Furthermore, we demonstrated that the metabolic benefits of hypothalamic PTP1B deficiency are dependent upon functional leptin receptor signaling, indicating that PTP1B's regulation of body weight and metabolism is mediated through leptin signaling within the hypothalamus. Finally, in chapter 4, we explored whether PTP1B could regulate IL-6 signaling, a non-leptin cytokine implicated in the central control of energy balance. Using an immortalized mouse hypothalamic cell line, we demonstrated that IL-6-stimulated STAT3 signaling does not appear to be regulated by endogenous PTP1B, though overexpression of PTP1B suppresses STAT3 activation. Taken together, these data highlight the continued importance of leptin as an adiposity signal that controls energy balance and confirm PTP1B's role as a negative regulator of central leptin signaling.

Anatomic localization of PTP1B's metabolic effects

Through the use of genetic mouse models, we have narrowed down the anatomic and cell-type specificity of PTP1B's metabolic effects. While whole body or whole brain PTP1B-deficiency yields an improved, lean metabolic phenotype (Elchebly et al., 1999; Klaman et al., 2000; Bence et al., 2006), peripheral loss of PTP1B does not affect energy balance (Delibegovic et al., 2007, 2009; Owen et al., 2012; Grant et al., 2013), indicating that PTP1B's metabolic contribution is localized to the CNS. POMC neuron-specific PTP1B knockouts demonstrate that PTP1B within POMC neurons also contributes to regulation of energy balance, however, compared to whole brain PTP1B knockouts, POMC neuron-specific PTP1B deficient mice show relatively minor improvements in body weight on HFD and no measurable differences when fed a chow diet (Banno et al., 2010). In chapter 2, we demonstrate that PTP1B deficiency within LepRb-expressing neurons largely recapitulates the metabolic phenotype of HFD-fed whole body PTP1B knockouts, and like whole brain PTP1B knockouts show decreased body weight even when fed a chow diet (Tsou et al., 2012). Figure 3.7 in chapter 3 schematizes the deletion sites of all CNS PTP1B-deficient models to date. In this diagram, one can see that POMC neurons represent a small subset of LepRb-expressing neurons, let alone the whole brain. Because of this relatively constrained central POMC expression, the weaker metabolic phenotype is representative of limited central PTP1B deletion. The remaining PTP1B expression in neuron populations excluding POMC neurons likely accounts for the milder phenotype. In chapter 3, we generate a hypothalamic PTP1B-deficient mouse model. Given that the hypothalamus is a major control center for energy homeostasis and hypothalamic LepRb deficiency results in a strong obese phenotype (Ring and Zeltser, 2010), we were surprised to see that hypothalamic PTP1B knockouts display a relatively modest lean phenotype compared to that of whole body, whole brain, and LepRb-expressing cell-specific PTP1B-deficient models. Taken together, the findings from these CNS PTP1B deficient models suggest that the majority of PTP1B's central metabolic contribution is localized to LepRb-expressing neurons, and PTP1B’s metabolic effects are localized to both hypothalamic and extrahypothalamic LepRb-neuron populations.

Several extrahypothalamic LepRb sites have been shown to affect energy balance. For example, hindbrain LepRb neurons are also required for normal energy balance (Hayes et al., 2010), and there is evidence of PTP1B's regulation of hindbrain leptin signaling (De Jonghe et al., 2012). Furthermore, hippocampal LepRb have been implicated in control of food intake and food-related memory. Direct injection of leptin into the ventral hippocampus suppresses food intake in rats and blocks expression of appetitive conditioned place preference (Kanoski et al., 2011); however, cortex and hippocampal PTP1B-defiency ($PTP1B^{fl/fl}$-Emx1-Cre) does not result in any metabolic phenotype (Fuentes et al., 2012 and unpublished data), suggesting enhanced hippocampal leptin sensitivity may not appreciably affect total energy balance. LepRb is also expressed in dopaminergic neurons of the midbrain ventral tegmental area (VTA) (Figlewicz et al., 2003). Direct VTA leptin administration suppresses food intake, and knockdown of VTA LepRb in rats results in increased sucrose preference and an increase in HFD intake, suggesting VTA leptin plays a role in hedonic energy intake (Hommel et al., 2006). Could VTA PTP1B expression modulate reward value associated with palatable food intake? Examining whether or not PTP1B can also regulate energy balance in these LepRb-expressing regions would further hone in on its exact CNS sites of action.

PTP1B and the central circuits regulating energy balance

The hypothalamus has long been regarded as a major CNS region controlling energy balance. Hypothalamic lesion studies in the early-mid 20th century and Kennedy's proposed endocrine feedback model have paved our understanding of the central control of energy homeostasis (Brooks, 1948; Kennedy, 1951, 1953). With the discovery of central leptin signaling and melanocortinergic pathways in the hypothalamus, a hypothalamus-centric model of energy balance has developed wherein first order, leptin-sensing neurons in the arcuate nucleus detect adiposity signals from the periphery and project directly to second order neurons as well as indirectly to neurons (hypothalamic or extrahypothalamic) further downstream. Within this

framework, PTP1B deletion (in LepRb-expressing cells for example) would sensitize leptin signaling within first order neurons, resulting in greater inhibition of AgRP neurons and enhanced activation of melanocortin neurons as well as downstream second order neurons involved in catabolic effects on energy balance.

More recently, the limitations of the hypothalamus-centric model of central energy homeostasis regulation have become apparent with its simplified unidirectional flow of information. If circuits beginning at the level of the hypothalamus were the primary regulators of energy balance, then we would have expected a more robust metabolic effect of hypothalamic PTP1B deletion as sensitization at the start of the circuit would presumably enhance downstream neuron activity. The relatively modest body weight improvements of deleting PTP1B in first order hypothalamic neurons lend support for a more distributed model of CNS energy balance control wherein first order neurons are located at different sites across the brain, and broad interconnectivity between these sites (hypothalamus, midbrain, hindbrain) results in coordinated processing of peripheral energy status signals (Grill and Hayes, 2012). Indeed, the CNS appears to receive energy status input from the periphery via multiple sources (blood-borne signals to both the hypothalamus and hindbrain, vagal afferents to hindbrain) and involves numerous interconnected regions including the aforementioned extrahypothalamic LepRb-expressing sites in addition to the hypothalamus. The enhanced hindbrain leptin sensitivity in POMC-PTP1B deficient mice (De Jonghe et al., 2012) suggests PTP1B can regulate energy balance at the level of peripheral input to the hindbrain NTS in addition to the arcuate nucleus of the hypothalamus. Furthermore, the relatively robust metabolic improvements seen in LepRb-PTP1B-/- mice when compared to the moderately improved body weight of Nkx2.1-PTP1B-/- mice suggest anatomically distributed central PTP1B-deficiency can enhance CNS catabolic energy balance processing.

Accounting for early development

The findings from chapters 2 and 3, as well as past PTP1B-deficient mouse models, demonstrate that genetic deletion of PTP1B results in an improved metabolic phenotype, but all models described to date have early loss of PTP1B at embryonic stages, leaving open the possibility that changes during development account for the metabolic benefits of PTP1B deficiency rather than a direct loss of gene/protein function. Two questions come to mind: could early PTP1B deficiency be changing developing feeding/metabolic circuits, resulting in the observed phenotypes? Moreover, can one produce metabolic improvements if PTP1B is deleted or inhibited in adults, once developmental programming has completed? Leptin deficiency has been shown to profoundly affect the development of hypothalamic circuits; *ob/ob* mice display permanent disruptions to arcuate-paraventricular nucleus projections which cannot be rescued in adulthood (Bouret and Simerly, 2004; Bouret et al., 2004). Could early leptin sensitivity elicited by PTP1B deletion enhance leptin's trophic effects producing an overabundance of arcuate neuronal projections? Gross brain morphology is normal in CNS PTP1B-deficient models, and no changes were observed in α-melanocyte-stimulating hormone labeled projections to the PVN in the brains of POMC-PTP1B-/- mice (Banno et al., 2010). Additional research is warranted to further explore whether early loss of PTP1B can affect trophic signaling or possibly neuron activity, leading to changes in CNS circuit development.

Whether or not adult PTP1B deficiency can bring about metabolic improvements is currently unknown, though recent evidence supports this idea. Adult diet-induced obese rats with third ventricle administration of PTP1B anti-sense oligonucleotides demonstrate decreased body weight, adiposity, and food intake, and increased central leptin and insulin sensitivity compared to rats treated with PTP1B sense oligonucleotides (Picardi et al., 2008). Furthermore, pharmacological inhibition of PTP1B in 20-week old, leptin-resistant rats enhances central leptin-induced food intake suppression (Morrison et al., 2007). To further explore the metabolic effects of adult PTP1B deficiency, our lab is currently using stereotaxic injections of AAV (adeno-

associated virus) driving Cre expression into adult diet-induced obese PTP1B fl/fl mice. Using this method, we can target PTP1B for deletion at any age and examine whether central loss of PTP1B can rescue diet-induced weight gain *in vivo* after the fact. Animals will be compared to age-matched control mice injected with AAV expressing GFP. Because this method is based on stereotaxic coordinates, we can further explore the contribution of different hypothalamic and extrahypothalamic nuclei at different developmental time points. Additionally, our lab is currently exploring the use of inducible-Cre mouse lines (Feil et al., 2009) as another alternative method to generate PTP1B mutants with tight temporal and spatial control.

Models of leptin resistance and the role of PTP1B in the pathogenesis of obesity

Whether leptin resistance is an underlying cause or consequence of obesity remains unclear; however, cellular leptin resistance can increase susceptibility to obesity and likely contributes to weight gain. Several mechanisms of cellular leptin resistance have been proposed, including decreased leptin transport across the blood brain barrier and dysfunction of leptin receptor signaling at the molecular/cellular level (Myers et al., 2012). Indeed, HFD-feeding has been shown to diminish leptin transport and increase expression of SOCS-3 and PTP1B (Bjørbaek et al., 1999; Levin et al., 2004; White et al., 2009; Lou et al., 2010). The mechanisms by which over-nutrition provoke increased expression of leptin's negative regulators remains unclear. Increased food intake and associated adiposity leads to elevated circulating leptin levels, and leptin has been shown to induce both SOCS-3 and PTP1B expression (Bjørbaek et al., 1999; White et al., 2009). Therefore, increased feedback inhibition in this context may promote leptin resistance. Alternatively, increased dietary fat increases circulating saturated fatty acids which are known to activate hypothalamic inflammatory pathways implicated in disrupted leptin signaling. For example, toll-like receptor 4 (TLR4) in the brain can be activated by long-chain saturated fatty acids (Moraes et al., 2009), and activity of the downstream serine kinase IKKβ/nuclear factor-κB (NF-κB) has been linked to leptin resistance via increased SOCS-3 expression (Zhang et al., 2008). Furthermore, palmitate has been shown to induce PTP1B

expression in skeletal muscle cells via activation of the IKKβ/NF-κB pathway, raising the interesting possibility that a similar mechanism may occur in the brain (MohammadTaghvaei et al., 2012). In addition to increased leptin levels and circulating fatty acids, long-term high-fat feeding can lead to hyperglycemia. Little is known about the role of central PTP1B in neuronal glucose sensing, although high-fructose feeding has been implicated in the development of hypothalamic leptin resistance (Bursać et al., 2014) and has been shown to induce PTP1B activity and expression within the liver (Li et al., 2010).

Although central PTP1B deletion can clearly sensitize leptin signaling resulting in a lean metabolic phenotype, whether or not PTP1B actively plays a role in the development of obesity pathology is less clear. One possible way to test this would be to generate mice with tissue-specific PTP1B overexpression (OE). We hypothesize that CNS-targeted PTP1B OE would yield susceptibility to obesity and increased weight gain. To generate CNS site-specific PTP1B OE, one could generate a line of transgenic mice with a floxed-stop cassette upstream of a wildtype *Ptpn1* transgene driven by a constitutive promoter. These stop-floxed PTP1B OE mice could subsequently be crossed with LepRb-Cre or Nkx2.1-Cre mice to determine if PTP1B OE exclusively within LepRb-expressing cells or the hypothalamus can produce an obese phenotype. Overexpression of human PTP1B in muscle leads to impaired insulin signaling and whole body insulin resistance in mice (Zabolotny et al., 2004); therefore PTP1B OE in LepRb-expressing cells or the hypothalamus could potentially lead to site-specific leptin resistance. On the other hand, while SOCS-3 OE in POMC neurons results in increased body weight and adiposity, SOCS-3 OE in LepRb-expressing cells yields an unexpected lean phenotype (Reed et al., 2010). Careful examination of PTP1B OE in different CNS sites will be necessary to determine whether it plays a pathological role in leptin resistance and obesity.

Confirmation of non-leptin pathway regulation

In chapter 3, we determined that the metabolic effects of hypothalamic PTP1B deficiency require intact leptin signaling and discussed the possibility of two models of PTP1B's central regulation of energy balance: (a) PTP1B's metabolic role in the CNS is exclusively as a negative regulator of leptin signaling, and the metabolic benefit of PTP1B deficiency is solely the result of enhanced leptin sensitivity, or (b) PTP1B acts on multiple signaling pathways (e.g. insulin, non-leptin cytokines, growth factors etc.) including leptin, and the metabolic effects of PTP1B deficiency are a combination of numerous sensitized pathways of which leptin is the major contributor. The latter model seems more likely given PTP1B's known regulation of insulin signaling and the evidence of enhanced hypothalamic insulin signaling and insulin-induced food intake suppression with CNS PTP1B knockdown via anti-sense oligonucleotides (Picardi et al., 2008). However, the insulin and leptin pathways have also been shown to have the potential for significant crosstalk through PI3K activation, which has been implicated in pharmacological food intake suppressive effects (Niswender and Schwartz, 2003; Niswender et al., 2003; Belgardt and Brüning, 2010). To further examine whether CNS PTP1B can regulate non-leptin signaling pathways, we can begin by looking at whether PTP1B deficiency affects insulin-induced anorexia or PI3K-activation. Insulin can be administered via third ventricle ICV into Nkx2.1-LepRb$^{-/-}$ and Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice, and food intake and markers of insulin signaling can be measured to see if Nkx2.1-PTP1B$^{-/-}$:LepRb$^{-/-}$ mice display enhanced insulin sensitivity relative to Nkx2.1-LepRb$^{-/-}$ mice.

Furthermore, the PTP1B:LepRb double mutant studies from Chapter 3 can be repeated using a pan-neuronal Cre line to examine whether or not total CNS PTP1B has leptin-independent metabolic effects. Comparing Nkx2.1-PTP1B$^{-/-}$ mice to previous PTP1B-deficient mice suggests that both hypothalamic and extrahypothalamic PTP1B contribute to its metabolic effects. Repeating this study is not without its challenges however, as currently available pan-

neuronal Cre lines each have their own disadvantages (e.g. Nestin-Cre and Synapsin-Cre) and would require the generation of large animal cohorts with thorough controls (Harno et al., 2013).

We demonstrated in chapter 4 that endogenous PTP1B does not appear to regulate IL-6-stimulated JAK-STAT signaling in mHypoE-N46 cells. Further study is required to map out which janus kinase, if any, is responsible for STAT3 activation in mHypoE-N46 cells. Additionally, we found that PTP1B overexpression suppresses IL-6-stimulated STAT3 activation in mHypoE-N46 cells. Given that increased hypothalamic PTP1B expression is associated with HFD-feeding and TNFα administration (Zabolotny et al., 2008; White et al., 2009; Picardi et al., 2010; Ito et al., 2012), hypothalamic IL-6 signaling could be further explored in both *in vitro* and *in vivo* models of nutrient excess and/or increased inflammation. Interestingly, IL-6 has been shown to suppress hypothalamic PTP1B expression (Chiarreotto-Ropelle et al., 2013), suggesting the possibility of reciprocal regulation. Additional studies are required to determine exactly how hypothalamic PTP1B can regulate IL-6 signaling, and vice versa.

PTP1B as a target for therapy: potential and challenges

Because of its role as a negative regulator of leptin and insulin signaling, PTP1B has become an attractive target for potential pharmacological intervention in the treatment of obesity and type 2 diabetes. A variety of PTP1B genetic mouse models, including the ones described in this thesis, demonstrate that limiting PTP1B's function could potentially lead to improved metabolic outcomes. While genetic deletion of *Ptpn1* in mice produces profound metabolic phenotypes, the metabolic benefits of pharmacological inhibition of PTP1B in humans are unclear; however, findings from whole body, whole brain, LepRb-expressing cell-specific, and hypothalamic PTP1B+/- mouse models suggest that body weight improvements can even be conferred by partial loss of function (Klaman et al., 2000; Bence et al., 2006; Tsou et al., 2012).

Thus, designing small molecules to inhibit PTP1B is a compelling approach with great potential towards treating and preventing metabolic syndrome and its associated disorders.

Developing highly-specific drugs targeted against PTP1B is not without its challenges. PTPs can regulate different pathways dependent upon tissue or cellular context. For example, PTP1B has been demonstrated to regulate growth factor-activated pathways in which loss of function could have severe, deleterious tumorigenic effects. PTP1B is a negative regulator of epidermal growth factor receptor and platelet-derived growth factor receptor pathways (Flint et al., 1997; Liu and Chernoff, 1997). Interestingly, PTP1B can play either a tumor-suppressor or tumorigenic role (Yip et al., 2010). Loss of PTP1B in tumor-prone p53 mutants results in decreased survivability and increased susceptibility to tumor formation (Dubé et al., 2005). In contrast, in human cases of breast cancer, PTP1B protein levels are elevated (Wiener et al., 1994), and loss of PTP1B in mice susceptible to mammary tumor growth led to delays in tumor onset (Bentires-Alj and Neel, 2007; Julien et al., 2007). Therefore, safe and effective PTP1B inhibitors require exquisite anatomic/cellular specificity in order to prevent potential tumorigenic side effects while permitting the desired, beneficial metabolic outcomes.

Separate from its role in cancer, PTP1B also plays a role in non-metabolic CNS functions which could yield potential side effects of drug action. PTP1B expression has been shown to affect hippocampal dendritic spine morphology. Interestingly, hippocampus and cortex-specific PTP1B-deficient mice show an enhancement in learning and memory tasks (Fuentes et al., 2012), suggesting that PTP1B inhibition may prove fruitful outside of enhancing CNS leptin signaling. Past reports demonstrate leptin and insulin having opposing effects on POMC neuron excitability (Cowley et al., 2001; Choudhury et al., 2005; Plum et al., 2006; Williams et al., 2010); PTP1B deficiency, therefore, could not only affect the sensitivity of intracellular signaling, but also neuron activity. Future research is essential for clarifying the metabolic *and* non-metabolic CNS effects of PTP1B in order to advance effective and safe therapeutic development.

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